

THE ROTHAMSTED
MEMOIRS
ON
AGRICULTURAL SCIENCE

VOLUME XIX
1933-1935

ST. ALBANS
GIBBS & BAMFORTH LTD., SPENCER STREET
1936

The Rothamsted Memoirs on Agricultural Science

CROPS, PLANT GROWTH, PLANT PRODUCTS, ACTION OF MANURES

CROPS

	Published
1. "The Future of British Agriculture." E. J. RUSSELL. Address to the Royal Institution of Great Britain	1935
2. "The Climatic and Soil Requirements of Tea." H. H. MANN. Empire Jour. Experimental Agric., Vol. I, pp. 245-252	1933
3. "The Influence of Season and of the Application of Lime on the Botanical Composition of Grassland Herbage." W. E. BRENCHEY. Ann. App. Biol., Vol. XXII, pp. 183-207	1935
4. "Mixed Cropping in Primitive Agriculture." H. NICOL. Empire Jour. Experimental Agric., Vol. III, pp. 189-195	1935

PLANT GROWTH

5. "The Influence of Rainfall on the Yield of Mangolds at Rothamsted." R. J. KALAMKAR. Jour. Agric. Sci., Vol. XXIII, pp. 571-579	1933
6. "The Effect of Rubidium Sulphate and Palladium Chloride on the Growth of Plants." W. E. BRENCHEY. Ann. App. Biol., Vol. XXI, pp. 398-417	1934
7. "Studies in the Absorption of Calcium from Nutrient Solutions with Special Reference to the Presence or Absence of Boron." K. WARINGTON. Ann. Bot., Vol. XLVIII, pp. 743-776	1934
8. "The Effect of Sodium Nitrate on the Growth and Nitrogen Content of a Lucerne and Grass Mixture." H. G. THORNTON and H. NICOL. Jour. Agric. Sci., Vol. XXIV, pp. 269-282	1934
9. "The Derivation of the Nitrogen of Crop Plants, with Special Reference to Associated Growth." H. NICOL. Biolog. Rev., Vol. IX, pp. 383-410	1934

10. "Further Evidence upon the Nitrogen Uptake of Grass Grown with Lucerne." H. G. THORNTON and H. NICOL. Jour. Agric. Sci., Vol. XXIV, pp. 540-543 1934
11. "Some Effects of Clipping the Tops upon the Root Development of Lucerne (*Medicago Sativa* L.)." H. G. THORNTON and H. NICOL. Jour. Agric. Sci., Vol. XXIV, pp. 532-539 1934
12. "Yield, Duration, and Drought-Resistance of Lucerne as Influenced by Frequency and Time of Cutting." H. NICOL. Empire Jour. Experimental Agric., Vol. II, pp. 380-390 .. 1934
13. "The Effect of Environmental Conditions upon Pyrethrum (*Chrysanthemum Cinerariaefolium*). I." J. T. MARTIN and F. TATTERSFIELD. Ann. App. Biol., Vol. XXI, pp. 670-681 1934
14. "The Effect of Environmental Conditions upon Pyrethrum (*Chrysanthemum Cinerariaefolium*). II." J. T. MARTIN and F. TATTERSFIELD. Ann. App. Biol., Vol. XXI, pp. 682-690 1934

PLANT PRODUCTS

15. "A New Method for the Determination of Cellulose, based upon Observations on the Removal of Lignin and Other Encrusting Materials." A. G. NORMAN and S. H. JENKINS. Biochem. Jour., Vol. XXVII, pp. 818-831 1933
16. "The Determination of Lignin. I. Errors Introduced by the Presence of Certain Carbohydrates." A. G. NORMAN and S. H. JENKINS. Biochem. Jour., Vol. XXVIII, pp. 2147-2159 1934
17. "The Determination of Lignin. II. Errors Introduced by the Presence of Proteins." A. G. NORMAN and S. H. JENKINS. Biochem. Jour., Vol. XXVIII, pp. 2160-2168 1934
18. "The Determination of Amino-Acids in Wheat Flour." L. W. SAMUEL. Biochem. Jour., Vol. XXVIII, pp. 273-282 1934

ACTION OF MANURES

19. "The Decomposition of Green Manures in Soil." J. A. DAJI. Jour. Agric. Sci., Vol. XXIV, pp. 15-27 1934
20. "The Fertilising Value and Nitrifiability of Humic Materials Prepared from Coal." E. M. CROWTHER and W. E. BRENCHLEY. Jour. Agric. Sci., Vol. XXIV, pp. 156-176 .. 1934
21. "Studies on Calcium Cyanamide. IV. The Use of Calcium Cyanamide and Other Forms of Nitrogen in Grassland." H. L. RICHARDSON. Jour. Agric. Sci., Vol. XXIV, pp. 491-510 1934
22. "Studies on Calcium Cyanamide. V. The Utilisation of Calcium Cyanamide in Pot Culture Experiments." H. L. RICHARDSON and E. M. CROWTHER. Jour. Agric. Sci., Vol. XXV, pp. 132-150 1935

STATISTICAL METHODS AND RESULTS

MATHEMATICAL THEORY

Published

23. "The Formation of Latin Squares for Use in Field Experiments." F. YATES. *Empire Jour. Experimental Agric.*, Vol. I, pp. 235-244 1933
24. "The 6×6 Latin Squares." R. A. FISHER and F. YATES. *Proc. Cambridge Phil. Soc.*, Vol. XXX, pp. 492-507 .. 1934
25. "The Analysis of Multiple Classifications with Unequal Numbers in the Different Classes." F. YATES. *Jour. American Statistical Association*, Vol. XXIX, pp. 51-66 .. 1934
26. "Contingency Tables Involving Small Numbers and the χ^2 Test." F. YATES. *Supplement to Jour. Roy. Statistical Soc.*, Vol. I, pp. 217-235 1934

TECHNIQUE OF FIELD EXPERIMENTS

27. "The Value of a Uniformity Trial in Field Experimentation with Rubber." R. K. S. MURRAY. *Jour. Agric. Sci.*, Vol. XXIV, pp. 177-184 1934
28. "Observer's Bias in Sampling-Observations on Wheat." F. YATES and D. J. WATSON. *Empire Jour. Experimental Agric.*, Vol. II, pp. 174-177 1934
29. "The Border Effect in Plot Experiments." A. V. COOMBS. *Empire Jour. Experimental Agric.*, Vol. II, pp. 315-323 .. 1934
30. "A Complex Pig-Feeding Experiment." F. YATES. *Jour. Agric. Sci.*, Vol. XXIV, pp. 511-531 1934

THE SOIL

SOIL CLASSIFICATION

31. "Some Inductive Methods in Pedology." E. M. CROWTHER. *Trans. Third International Congress of Soil Sci.*, Vol. I, pp. 339-343 1935

SOIL CULTIVATION

32. "The Functions of Mechanical Power in Soil Cultivation." B. A. KEEN. *Proc. Institution Automobile Engineers*, Vol. XXIX, pp. 179-194 1935

PHYSICAL PROPERTIES

33. "The Effects of Rainfall and Temperature on Percolation through Drain Gauges." R. S. KOSHAL in consultation with R. A. FISHER. *Jour. Agric. Sci.*, Vol. XXIV, pp. 105-135 1934
34. "The Application of Heat of Wetting Measurements to Soil Research Problems." H. JANERT. *Jour. Agric. Sci.*, Vol. XXIV, pp. 136-150 1934

35. "On the Measurement of Imbibitional Water." E. W. RUSSELL and R. S. GUPTA. Jour. Agric. Sci., Vol. XXIV, pp. 315-325 1934
36. "The pF of the Water in Soil." R. K. SCHOFIELD. Trans. Third International Congress of Soil Sci., Vol. II, pp. 37-48 1935

PHYSICO-CHEMICAL PROPERTIES

37. "The Use of the Glass Electrode in Soil Reaction and Oxidation-Reduction Potential Measurements." S. G. HEINTZE. Jour. Agric. Sci., Vol. XXIV, pp. 28-41 1934
38. "Soil Oxidation-Reduction Potentials and pH Values." S. G. HEINTZE. Soil Research, Vol. IV, pp. 351-355.. .. 1935

CHEMICAL ANALYSIS

39. "An Examination of the Degtjareff Method for Determining Soil Organic Matter, and a Proposed Modification of the Chromic Acid Titration Method." A. WALKLEY and I. ARMSTRONG BLACK. Soil Science, Vol. XXXVII, pp. 29-38 1934
40. "An Examination of Methods for Determining Organic Carbon and Nitrogen in Soils." A. WALKLEY. Jour. Agric. Sci., Vol. XXV, pp. 598-609 1935

SOIL ORGANISMS

BACTERIA

41. "The Numbers of Bacterial Cells in Field Soils, as Estimated by the Ratio Method." H. G. THORNTON and P. H. H. GRAY. Proc. Roy. Soc., B, Vol. CXV, pp. 522-543 1934

PROTOZOA

42. "The Effect of Bacterial Products on Amoebic Growth." D. W. CUTLER and L. M. CRUMP. Jour. Experimental Biol., Vol. XII, pp. 52-58 1935

BIOLOGICAL ACTION

43. "The Biological Decomposition of Plant Materials. Part IX. The Aerobic Decomposition of Hemicelluloses." A. G. NORMAN. Ann. App. Biol., Vol. XXI, pp. 454-475 1934
44. "The Biological Oxidation of Carbohydrates. IV. The Phosphorus Requirements of Percolating Filters." S. H. JENKINS. Biochem. Jour., Vol. XXIX, pp. 116-132 .. 1935
45. "The Preferential Utilization of Different Forms of Inorganic Nitrogen in the Decomposition of Plant Materials." E. H. RICHARDS and J. G. SHRIKHANDE. Soil Science, Vol. XXXIX, pp. 1-8 1935

46. "A Study of the Composition and Utilisation of Alberta Peats." J. D. NEWTON. Ann. App. Biol., Vol. XXI, pp. 251-266 1934

THE PLANT IN DISEASE : CONTROL OF DISEASE

INSECT PESTS AND THEIR CONTROL

47. "Some Biological and Economic Aspects of the Gall Midges." H. F. BARNES. Science Progress, Vol. XXIX, pp. 73-86 1934
48. "A Cambium Miner of Basket Willows (Agromyzidae) and its Inquiline Gall Midge (Cecidomyiidae)." H. F. BARNES. Ann. App. Biol., Vol. XX, pp. 498-519 1933
49. "Studies of Fluctuations in Insect Populations. III. The Gall Midge, *Rhabdophaga Heterobia* H.L.W., on Black Maul Variety of *Salix Triandra* at Syston, Leicestershire, 1927-33." H. F. BARNES. Jour. Animal Ecology, Vol. III, pp. 165-181 1934
50. "On the Biology of Some Species of *Longitarsus* (Col., Chrysom.) Living on Ragwort." H. C. F. NEWTON. Bull. Entomological Research, Vol. XXIV, pp. 511-520 1933
51. "On the Biology of *Psylliodes Hyoscyami* Linn. (Chrysomelidae, Coleoptera), the Henbane Flea-Beetle, with Descriptions of the Larval Stages." H. C. F. NEWTON. Ann. App. Biol., Vol. XXI, pp. 153-161 1934
52. "The Loss of Activity of Pyrethrum. II." F. TATTERSFIELD and J. T. MARTIN. Jour. Agric. Sci., Vol. XXIV, pp. 598-626 1934
53. "An Apparatus for Testing Contact Insecticides." F. TATTERSFIELD. Ann. App. Biol., Vol. XXI, pp. 691-703 1934

BACTERIAL DISEASES

54. "An Experiment on the Incidence and Spread of Angular Leaf-Spot Disease of Cotton in Uganda." C. G. HANSFORD, H. R. HOSKING, R. H. STOUGHTON and F. YATES. Ann. App. Biol., Vol. XX, pp. 404-420 1933
55. "The Influence of Environmental Conditions on the Development of the Angular Leaf-Spot Disease of Cotton. V. The Influence of Alternating and Varying Conditions of Infection." R. H. STOUGHTON. Ann. App. Biol., Vol. XX, pp. 590-611 1933

VIRUS DISEASES

56. "The Physiology of Virus Diseases in Plants. V. The Movement of the Virus Agent in Tobacco and Tomato." J. CALDWELL. Ann. App. Biol., Vol. XXI, pp. 191-205 1934

	Published
57. "The Physiology of Virus Diseases in Plants. VI. Some Effects of Mosaic on the Metabolism of the Tomato." J. CALDWELL. <i>Ann. App. Biol.</i> , Vol. XXI, pp. 206-224 ..	1934
58. "The Physiology of Virus Diseases in Plants. VII. Experiments on the Purification of the Virus of Yellow Mosaic of Tomato." J. CALDWELL. <i>Ann. App. Biol.</i> , Vol. XXII, pp. 68-85	1935
59. "A Comparison of English and Canadian Tomato Virus Diseases." G. C. AINSWORTH, G. H. BERKELEY and J. CALDWELL. <i>Ann. App. Biol.</i> , Vol. XXI, pp. 566-580 ..	1934
60. "Experiments Bearing on the Nature of Intracellular Inclusions in Plant Virus Diseases." F. M. L. SHEFFIELD. <i>Ann. App. Biol.</i> , Vol. XXI, pp. 430-453	1934

MISCELLANEOUS

61. "The Constancy of the Viscosity of Strong Lithium Chloride Solutions at Low Velocity Gradients." G. W. SCOTT BLAIR and R. K. SCHOFIELD. <i>Phil. Mag.</i> , Vol. XVII, pp. 225-229	1934
62. "A Photographic Moonlight Recorder." C. B. WILLIAMS and G. A. EMERY. <i>Jour. Scientific Instruments</i> , Vol. XII, pp. 111-115	1935

ROYAL INSTITUTION OF GREAT BRITAIN
WEEKLY EVENING MEETING

Friday, March 8, 1935

LORD EUSTACE PERCY, P.C., M.A., M.P.

President, in the Chair

SIR JOHN RUSSELL, D.Sc., F.R.S.

Director of the Rothamsted Experimental Station

THE FUTURE OF BRITISH
AGRICULTURE

IN choosing this subject I am very mindful of the many eminent persons who first made some reputation by good work dealing with the present or the past, and then proceeded to lose it by dabbling in the future. But one must take some risks ; and in these critical times, when all sorts of experiments in agricultural organisation are being tried, one must sometimes venture to look ahead. It need not be pure speculation, the safer method is to study the present trends and movements, and see if it is possible to forecast their probable development.

Certain factors in the agricultural situation are unlikely to change. The natural conditions of Great Britain are very varied, and agriculture is, and will probably remain, not one industry but many. The dry eastern counties are pre-eminently arable, producing corn, potatoes, sugar beet, fruit and pigs. The great open Downs of the southern counties produce corn, sheep and milk. The grass vales of the Midlands produce meat, the middle-west produces milk, the far west, Wales and the North, produce animals. It is not very likely that these distinctions will ever disappear. With these varying regional activities

SIR JOHN RUSSELL

farmers' interests will always be divergent, and in trying to reconcile them every Minister of Agriculture will have a very anxious and difficult task.

Further, it seems likely that our present individual farming will persist, and that it will continue to be of the small capitalist type. At present out of our 25½ million acres of cultivated land* about half is in holdings ranging from 100 to 300 acres; this tends to shrink in favour of rather smaller holdings. Many attempts have been made to subdivide these and increase the small holdings (below 50 acres) but they have not proved successful. Occasionally wealthy individuals or large companies have set up to farm on the grand scale, throwing a number of farms into one, introducing all kinds of efficiency methods. Some have been very successful but many have failed; in the end most of the companies gave up the unequal struggle. The countryman too is likely to remain always the sturdy individualist he now is. In the War time and just after there was much state supervision of farming, but it failed, and many of us remember the chuckles of delight in the countryside when the newspapers announced "Another batch of farm inspectors axed." State farms failed in France in the Revolution, they have failed in Soviet Russia; indeed Russia with all its zeal for experiment and its complete power over the peasant has not yet discovered how to socialise agriculture successfully.

Whatever may happen to coal, to banking, to industry, we may assume that the countryman will always remain an incurable individualist.

* England and Wales, 1930, 25.380 million acres.

THE FUTURE OF BRITISH AGRICULTURE

Further, the English countryman will always differ from the townsman in his knowledge of and attachment to animals. In spite of the fact that the Englishman is always at heart a countryman wherever he lives, we may suppose that the townsman will know less and less about animals as the years go by ; that pigs, sheep and cows will become only dots and flitting shadows seen from a high-speed car or an aeroplane, and that horses will be only names on a betting slip. But for the English countryman animals will always be among his dominating interests. The production of animals and of milk will probably continue to form the major part of his occupation. The live-stock show, the hunt, the point-to-point race meeting, will probably always form his major amusements. With all this he is likely to retain his love for craftsmanship and his essential dislike of mass production methods. The smith still keeps his love of working in iron. The young men crowd round the village garage and welcome the chance of taking your car to pieces and reassembling it, which they do tolerably well. During the last 30 years rural education has been completely overhauled, and there is a continuous network of agencies, beginning with the school garden and the school talks given by experts and organised by the B.B.C., then working through the County Agricultural organisers, the County Farm Institutes and the Agricultural Colleges and Research Institutes under the Ministry of Agriculture, to ensure that all the resources of science shall be available for education and for use. These have already produced a generation of young farmers who are alert, keen and anxious to make the best of their farms and their workers ; indeed, a recent

SIR JOHN RUSSELL

economic inquiry in the Eastern Counties showed that the younger farmers had come better out of the recent economic troubles than the older ones. The demand for education in the country districts is greater than ever and we can look forward with considerable hope to the coming race of farmers and farm workers. They will be different from the townsmen, more independent and self reliant, certainly no less intelligent, and at least as good citizens.

TABLE I.—TIME NEEDED FOR FOOD-PRODUCTION
AS FROM APRIL 1ST.
FOR CROPS.

	Planted	Reaped	Months required
Wheat ...	Oct.—Dec.	July—Aug.	17
Potatoes ...	April	Oct.	7; more probably 19

FOR ANIMALS (MONTHS).

Milk ...	For Calf 9	Heifer 24	Gestation 9	Total 42
Beef ...	For Calf 9	Bullock 12–24		21–33 or more
Lamb ...	Born Jan.—Mar.	3–12		14–23
Bacon ...	For litter 4	Bacon pig 7		11

Another permanent factor in the situation is the slowness with which things move in agriculture.

THE FUTURE OF BRITISH AGRICULTURE

Crops can be sown only at certain times of the year, and they are in the ground for about 12 months. Animals take even longer to produce. If British farmers were asked to-morrow (March 9th, 1935) to double their output of wheat, of milk and of lambs, they could not do it for at least 18 months, it would be September or October, 1936, before the wheat would be threshed, the summer of 1938 when the lambs were ready, and some time in 1939 when the new cows began to give milk. Neither money nor science can speed these things up. Nature sets the pace, speed and volume of output are not under the same control as in industry.

English agriculture is exceedingly old, its roots go back at least 2,000 years; it has grown and developed continuously and this is likely to continue. New crops are always being tried. Only last year two of them, maize and soya beans, made quite a promising start. Whether we shall keep them we do not yet know. One fairly recent introduction, marrow stem kale, is proving exceedingly useful as food for animals. Even the animals are being changed, selected and bred for specific purposes. Mutton, for instance, is completely *démodé*, sheep for the butcher should all be lambs, and consequently farmers to-day want ewes that will produce twins every year. That has been done by selection from two prolific breeds, the Border Leicester and the Cheviots. We are trying to go a stage further by helping the ewes to produce more milk; usually they have only two teats, we have selected four-teated ewes and are breeding from them. This kind of change will go on, the farm animals of the future will change as the demands of the housewife change. We may

presume they will become younger and more tender, but as none can tell what the British housewife will want in future we will venture no forecast.

British agriculture has passed through two great phases and is now entering a third: we may call them increased production, cheaper production, and organised production. The forces that brought them about are still operating and seem likely to continue. Attempts have always been made to increase production but they developed remarkably rapidly from about 1850 onwards. The industrial revolution and the extraordinary and probably unique period of fecundity of the Victorian age, caused an unprecedented rise in the population and the demand for food. English farmers produced it. Lawes about 90 years ago showed how to make artificial fertilisers, beginning first in a barn fitted up as a laboratory at Rothamsted, then in a factory in London. The results were striking and at once appealed to farmers; within a few years yields had increased by about 30 per cent. Fertilisers were in growing demand and they played a large part in the development of modern Europe by freeing men's minds from the fear of hunger that had so long oppressed them. We now claim artificial fertilisers as a great triumph for science. A good case can be made, but if we are to be quite honest, we must remember that the great spokesman of science of those days, Liebig, roundly condemned the Rothamsted experiments and the fertiliser practices based on them :

“The experiments of Messrs. Lawes and Gilbert are very far indeed from proving the conclusions which they wish to draw; they establish rather the fact that these gentlemen have not the

THE FUTURE OF BRITISH AGRICULTURE

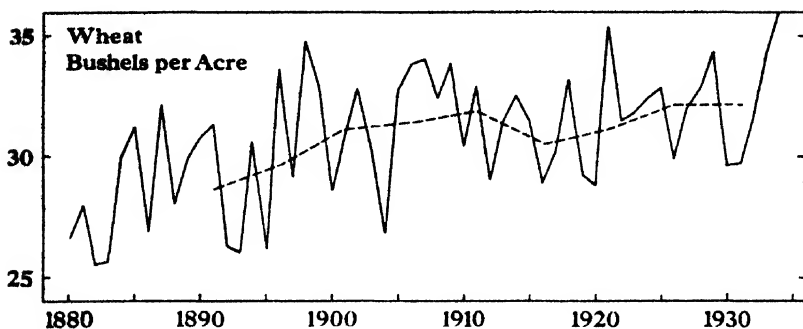


FIG. 1.—Yearly Yields of Wheat in Great Britain, 1880–1934.
----- 10 year means

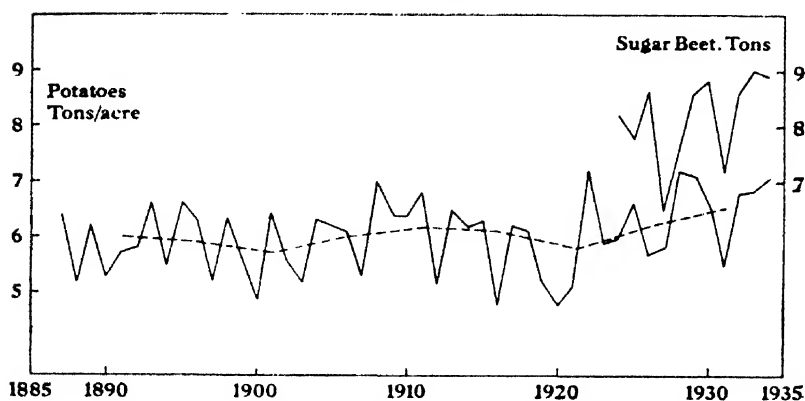


FIG. 2.—Yearly Yields of Potatoes, 1887–1934
and Sugar Beet 1924–1934, Great Britain.
----- 10 year means

slightest notion of what is meant by argument or proof.

“ If I here bestow upon these experiments an attention which they do not deserve, my object is, not to submit them to a passing criticism, but to warn the practical man how he ought not to proceed in trying to solve his problems.”

But the work developed so well that some 35 to 40 million tons of fertiliser are now made annually. The latest development is undoubtedly a

great achievement of science and engineering, the making of nitrogenous fertilisers from the nitrogen of the air, thereby giving inexhaustible supplies to ourselves and our children. The enormous factory at Billingham is capable of a far greater output than we are likely to use.

Other means of increasing the yields per acre have been developed: new implements, new varieties of crops, and better control of pests and diseases. Yields in this country are still rising, how far they will continue to do so I shall not attempt to forecast; for some years now the rise has been slow (Figs. 1 and 2) because farmers have been seeking to cheapen production rather than increase it per acre.

When the modern system of agriculture got into its stride in this country it so functioned that on an average 2 to $2\frac{1}{2}$ acres of land supported one person for a year. The population was still increasing. It would no doubt have been possible to increase yields per acre and to change methods so that less land per head would be needed. But for some 60 years or so another movement has been developing and it gathered great force before the War: it was to extend the area of land under cultivation. During the second half of the nineteenth century the United States, Canada and Australia were being opened up, vast areas of farm land were thrown open to settlement. In the 50 years between 1870 and 1920 no less than 450 million acres were added to the world's cultivated area, and the white population increased by 225 millions, an increase of 2 acres per head. Curiously enough this figure is about the same as the average area needed to provide the food per head of population in the cultivated countries:

THE FUTURE OF BRITISH AGRICULTURE

ACRES UNDER FOOD CROPS REQUIRED PER HEAD OF THE POPULATION (A. D. Hall).

England (T. H. Middleton's Estimates)	...	2-2.5
Germany	do.	1.3-1.5
Denmark (very efficient cultivation)	...	1.8
Exporting White Countries (excluding Russia)		2.4
United States	2.6
France	2.4
Spain (large areas of poor land)	4.0

Much of this new land, though inherently fertile, was for various reasons unsuited to existing varieties of plants, and new ones had to be found or bred, better suited to the local conditions than existing sorts. Our good English wheats, for instance, suffered from rust and frost in Canada; William Saunders and his sons at the Central Experimental Farm Ottawa therefore bred some new sorts, requiring much less time for growth and therefore ripening before rust could injure or frost kill. They produced Marquis, which, when recognised, rapidly spread through the prairies and opened up great possibilities for expansion; it is now grown more widely than any other sort of wheat in the world. It is the outstanding triumph of plant breeding—also often claimed as a triumph for science—but again we must confess that the strict sect of geneticists disowned the work at the outset, and quite recently I heard one of them deploring the fact that Marquis does not conform to the canons of genetical science, because if it did it would be such a splendid example of their practical value.

This rapid expansion of the area of cultivated land kept up with the growth of population and so

made it unnecessary to raise the yields per acre in England. For a time indeed questions of increased yields lost all interest, for the new countries had started another movement of which we now hear a great deal—mechanisation. They had always been short of man-power, their farmers were ingenious and adventurous and they took very kindly to machinery. On their great rolling plains, sparsely inhabited, with no obstacles in the form of gates, roads or hedges, the big machines had every chance to develop. Some amazing things have been produced especially for the cultivation of wheat. One vast machine will prepare the seed bed, and sow the wheat, leaving it so that nothing more need be done till harvest, when another great machine goes over the crop, cuts and threshes it in one operation, delivering the grain into wagons by which it can be carted to the bins or the elevators. The new machinery is very economical of labour. The ordinary English system of farming requires about 25 to 30 permanent men per square mile. On a highly mechanised Saskatchewan farm one man per square mile suffices. Ordinary English farming represents a higher efficiency per acre, but prairie mechanised farming represents a higher efficiency per man, the cost of production is therefore much less than on an English farm.

Unfortunately for the peace of mind of our farmers, engineers busied themselves with the improvement of transport, and they joined this up with cold storage of food and evolved systems of refrigerator transport. These have been so highly developed that butter, fruit, lamb and beef can be produced at one end of the world and sent for consumption to the other, and the ordinary person

THE FUTURE OF BRITISH AGRICULTURE

cannot tell the difference from the fresh home produce.

The result was an enormous increase in output from the great Dominions. (Fig. 3.) For the

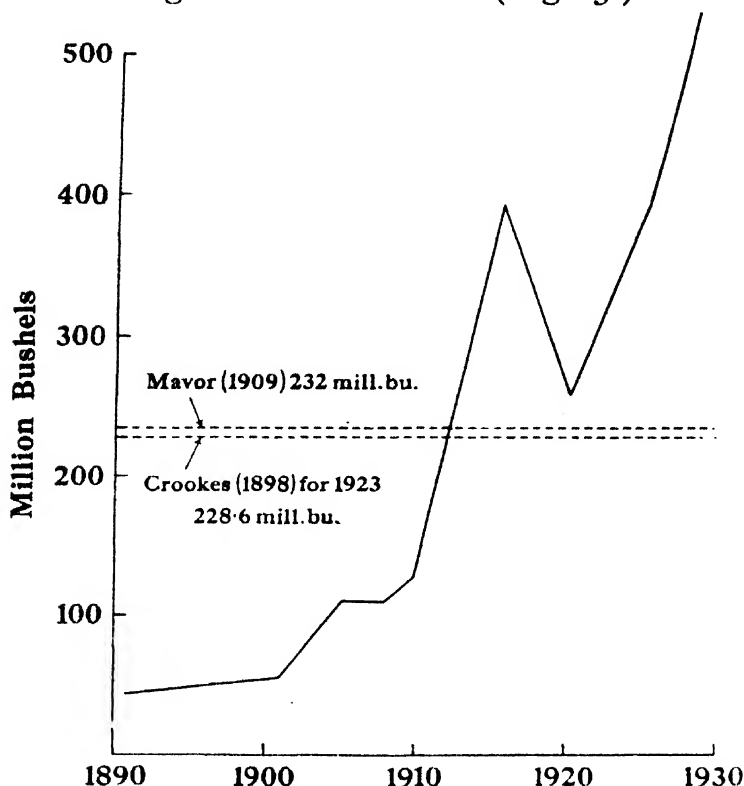


FIG. 3.—Canada's Wheat Production.

Crookes' and Mavor's forecasts of the probable limits of wheat production in Canada with the dates of forecasting are shown by dotted lines. William Saunders' forecast was 812 million bushels.

moment this extension of the area of cultivated land has stopped, partly as the result of the economic crisis, partly because of certain difficulties that have arisen in the continued cultivation of some of the prairie land. But all these can be overcome, and whenever the world is ready for it

larger areas of land can come into cultivation.

These then are the movements and factors dominating British agriculture at the present time : let us summarise them :—

(1) A considerable power of high production per acre, which is still rising though only slowly.

(2) A continued expansion of the world's area of cultivated land, suspended for the moment, but capable of resumption whenever needed.

(3) Great improvement in the methods of transport, so that farm produce can be brought from most parts of the world in such good condition that the ordinary British housewife can hardly tell the difference between home and imported food.

(4) In consequence fierce competition in our markets.

(5) At home, a race of efficient but strongly individualistic farmers, tied down to certain lines of production because they lack the capital to change, and tied down to certain rates of wages by the operations of the Agricultural Wages Boards.

Until quite recently every farmer produced whatever he thought fit, in whatever quantities he liked, in the joyous hope of a good market. He had no means of forecasting the demand for his produce or the price he would receive. There was nothing approaching a contract system ; in the manufacturers' phraseology the farmer produced only " for stock."

The net result of all these various factors has been a considerable glutting of the world's markets with food, and a collapse of prices, as shown in Table 2. Wheat prices fell particularly low, but even live stock, the farmer's sheet anchor, went down heavily in price.

THE FUTURE OF BRITISH AGRICULTURE

TABLE 2.

AVERAGE PRICES OF WHEAT AND MEAT, 1925 TO 1934. MINISTRY OF AGRICULTURE STATISTICS.

Year	General Agricltl. Produce	Price of Wheat per cwt.	Index	Price of Fat Cattle per live cwt.	Index	Price of Fat Sheep per lb. dead, <i>pence</i>	Index
1925	159	12/2	160	52/3	151	14 $\frac{3}{4}$	190
1926	151	12/5	164	48/8	141	12 $\frac{1}{4}$	157
1927	144	11/6	152	43/8	127	11 $\frac{1}{2}$	150
1928	147	10/-	132	47/9	138	12 $\frac{3}{4}$	167
1929	144	9/10	130	45/10	133	12 $\frac{1}{4}$	157
1930	134	8/-	105	45/6	133	12 $\frac{1}{2}$	160
1931	120	5/9	76	42/3	122	10 $\frac{1}{4}$	133
1932	112	5/11	78	39/7	115	7 $\frac{1}{2}$	97
1933	111*	{ 5/4 10/- ¹	{ 70 132 ²	{ 35/2	101	8 $\frac{1}{2}$	110
1934 ³	122†	{ 5/1 9/8 ¹	{ 67 128 ²	{ 33/8 38/5 ¹	{ 97 111 ²	9 $\frac{1}{2}$	123

* Allowing for wheat quota payment.

† Allowing for both wheat and cattle payments.

¹ Price including wheat quota payment, or bonus on fat cattle.

² Index calculated on the bonus price.

³ Last four months only.

Wages rates hardly altered during the time, in some countries there was a drop about 1931, in others not. Rents certainly fell and rates were abolished, but these form only a small part of the costs. The tragedy of the figures lies in the fact that the things sold this year started being produced at the prices ruling two years ago, and for ten years in succession farmers have been doing the initial operations at one level of price and selling the produce at a lower level. The natural consequence has been that they have consumed their own reserves and gone into debt to the banks and the merchants. (Fig. 4.)

SIR JOHN RUSSELL

Now agriculture is one of our largest industries ; it employs more people than most of the others and it keeps them so regularly in employment that until quite recently there was no widespread demand for Unemployment Insurance. If British agriculture went bankrupt we should be in for a first-class financial crisis. Moreover it has to be

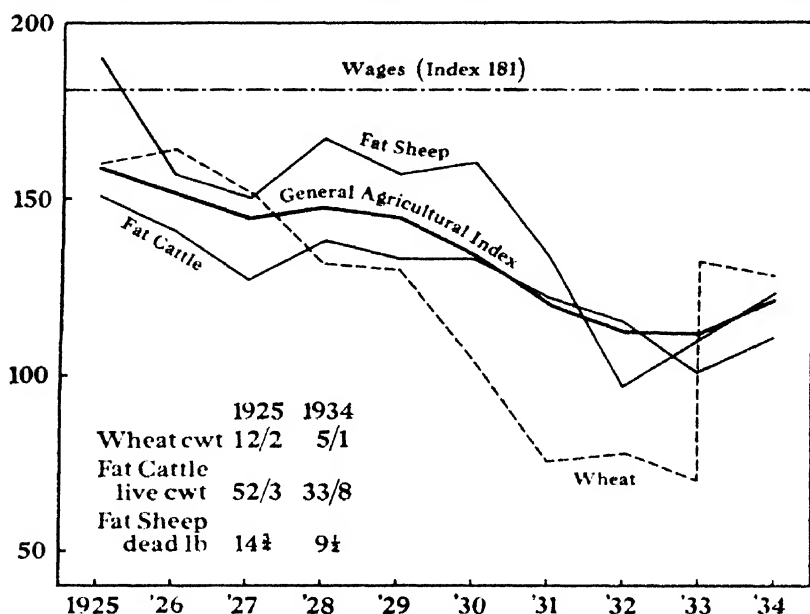


FIG. 4.—Agricultural prices 1925-34.
Index based on Average 1911-13 prices = 100.

kept functioning on grounds of National Health, for fresh milk must be delivered daily to every house where there are children, to say nothing of the need for fresh eggs, fresh vegetables, fresh fruit, and ignoring altogether possible value of freshness in meat and other foods. So something had to be done, and the Government introduced various devices for preventing further falls of prices and so keeping the industry going.

THE FUTURE OF BRITISH AGRICULTURE

There were two possible ways out. One was to increase consumption. That would certainly do something but it would not permanently solve the difficulty. Our population is just about stationary and will soon decline, so that no increase of food

TABLE 3.
AVERAGE FOOD SUPPLIES PER HEAD OF POPULATION.
GREAT BRITAIN.

	PRE-WAR (1907)	POST-WAR (Av. 1924-25 to 1927-28)
	lb.	lb.
Wheat Flour	208	207
Meat	144·9	147·1
Beef and Veal	69·4	71·4
Pigmeat and Lard	42·8	46·8
Mutton and Lamb	28·4	26·2
Fish	43·7	41·9
Poultry	3·9	3·7
Eggs (number)	111	116
Milk: Fresh (gallons)	19·5	20
Condensed and Powder (lb.)	—	8·1
Butter	15·8	15·4
Cheese	8·7	9·5
Margarine	5·0	12·4
Potatoes	189	192
Fruit and Nuts (all kinds)	74	101
Sugar	80	86
Cocoa	1·1	2·6

consumption can be expected like that occurring in the nineteenth century. Nor is there much likelihood of an increased consumption per head. In the matter of food, as every housewife knows, the Englishman is extraordinarily conservative. The War made a sharp break in our national life :

between Pre-War and Post-War lies a gulf that seems well-nigh unbridgeable. Yet our breakfasts, luncheons, dinners and teas have remained unchanged, the amounts of the different foods consumed per head of population are almost ludicrously similar. (Table 3.) More fruit is eaten and more sugar, margarine comes in as a new food, but the staple products show practically no change. And although a number of the population are recorded as wholly or partly unemployed, they are mostly in receipt of money from public and private sources, so that their income available for food is probably not less than many people had before the War. So far as the rest of the population are concerned, as people become better off they do not eat more food, many of them eat less.

Of course other countries do not consume anything like these amounts of food per head, and one might hope that they would increase their consumption. Many of them do not share our passion for slimming. But the resources of agricultural science are so great that no "Eat More Food" campaign, however seductive, would enable human beings to eat their way through the colossal mountains of food that the farmers of the world could produce if necessary.

The alternative is to regulate food production so as to bring it more nearly into line with consumption, allowing, of course, a liberal margin of safety. In principle this presents less difficulties than one might expect. Our total food requirements are pretty well known. Good estimates can be found of our own potential supplies, and of the exportable surpluses available from overseas.

Our total food requirements and the proportions

THE FUTURE OF BRITISH AGRICULTURE

grown at home, are given in Table 4. We produce on the average about 15 per cent of our wheat supply, about 44 per cent of our meat, 50 per cent of our dairy and poultry produce,

TABLE 4.

1933.

SOURCES OF FOOD FOR THE UNITED KINGDOM.

Commodity	Million			Per Cent		
	Home	Empire	Foreign	Home	Empire	Foreign
Wheat cwt.	33 ·4	75 ·3	37 ·2	23	52	25
Wheat + Imported Flour—as Wheat cwt.	33 ·4	82 ·3	40 ·0	22	53	25
Meat (excepting pig) cwt.	*11 ·6	7 ·3	11 ·6	38	24	38
Pig Meat, cwt.	*6 ·7	1 ·3	9 ·5	38	48	54
Eggs in Shell (millions)	4,727	727	1475	68	11	21
Milk gallons	*1,349	None	None	100		
Potatoes tons	5 ·5	0 ·08	0 ·12	97	1	2

* These figures are for England and Wales only, and are for 1932-33. All other figures refer to United Kingdom, and are for the 12 months, 1933.

practically all our potatoes and all our milk, and a large part of our fruit and vegetables, which, however, is not easily expressed as a percentage because no good estimate can be given of the large amount of vegetables produced in gardens and allotments. On the whole we produce about 35 to

40 per cent of our total food, and the rest is imported. It would be quite easy to produce more at home. We should put more workers on the land and they would give occupation to people in the villages. It looks a simple and obvious thing to do to bring together unemployed acres and unemployed men. But it is not quite so simple as it looks because of course all this imported food is paid for by goods or by services. If we produce more ourselves we should buy less from abroad, and so we should sell less abroad. In employing more people on our own land we should cut off the demand for someone else's labour.

It is commonly considered, and I shall not dispute it, that our present production of 36 to 40 per cent of our food supply is too low. It is equally admitted that if we produced 100 per cent of our food supply we should lose much of our export and shipping trade, besides raising a veritable hornet's nest among the Dominions representatives and our friendly European and American neighbours, which we definitely could not afford to do. The line is to be drawn somewhere in between—but where?

It would be a great advantage for all concerned if there could be an impartial inquiry by technical and business experts and representatives of the Ministry of Agriculture, the Foreign, Colonial and Dominions officers to advise where the line should be drawn. Should we aim at 50 per cent, which is quite a possibility, or should we aim even higher? I shall attempt no forecast here. What one can say is that the great body of British farmers compares very favourably with farmers anywhere else. This 35 to 40 per cent of our food, for instance, is produced by about a million farmers and

THE FUTURE OF BRITISH AGRICULTURE

workers, so that one agriculturist in England feeds about 20 persons. The corresponding figure for the United States is 12. For France it is less, but the actual figure is too low because it takes no account of exports, the value of which is complicated by the wine, which is more than a purely agricultural product. For Russia the figure is very much less. The comparisons are not exact because no account is taken of the work done by farmers' wives in Great Britain, so that our figure is somewhat overstated, but the relative order is probably correct.

For any ordered development of British agriculture it is essential to decide how much we should produce at home. This seems to require a contract system, with obligations on both sides, including the social obligation of paying a living wage to the workers. Several experiments in this direction are now being made. A straightforward contract is in use for milk, sugar beet, bacon, and a few minor things like seed and produce for canning. In these the farmer undertakes to produce and to deliver at stated times and at an agreed price, certain quantities of material conforming to defined standards. This arrangement works out satisfactorily for everyone. In the recent trying times the only bright spots have been milk, sugar beet, and bacon. The public does not suffer in any way, indeed it gains because contracts can stipulate higher quality. Unfortunately the method in this simple form applies only to commodities handled by a small number of large organisations, e.g., the Sugar Beet Factories, which can act as one Association, and the milk distributors, which in London are mainly one large concern. It is not applicable to commodities like

beef, mutton, potatoes, eggs, which are handled by a multitude of small distributors. For some of these, e.g., milk and bacon, Marketing Boards have been set up to act as clearing houses and ensure smooth adjustment between farmer and distributor. The livestock problem is not yet solved; in the meantime prices are kept from collapse by a temporary bonus system. Wheat always presents a delicate problem because of its special importance in our national dietary. An ingenious device was adopted which promises to be successful, and which could be applied to other farm products also. The 15 per cent we at present produce is to be paid for at a fixed rate of 10s. per cwt.; the excess price above market price is to be met from the 85 per cent of imported wheat. The arrangement is really a contract with farmers to buy from them an agreed quantity at an agreed price, but the arrangement is worked through the Wheat Board and not through the Government; there is no taxation of wheat. The low price of the imported wheat has prevented the price of bread from rising. For meat the same general idea is proposed but with the modification that the contract price to the farmer varies with the price of feeding stuffs, and the excess price over that of imported meat is to be met by a levy.

Experience will no doubt suggest modifications in procedure, and misunderstandings would certainly be avoided if some name could be invented for the payments of the agreed price to the farmers; at present they are sometimes represented as gifts or bonuses. Out of these various experiments there will undoubtedly emerge methods of contracting between the farmer and

THE FUTURE OF BRITISH AGRICULTURE

the community which will ensure that the community obtains its food at reasonable prices and enable the farmer to survive and to pay his men a living wage. Developments of the contract system will ensure the stability of British farming, and the machinery now in existence will ensure continued increase in efficiency of production and improvement of quality.

The canning industry is likely to play an increasing part in British agriculture, for undoubtedly the younger generation all over the country are more and more forsaking fresh food and living on canned produce. In absence of any very pronounced medical opinion against it a layman cannot speak of the effects on the consumer; for the farmer it has the advantage that the material is all grown on contract. Further, the factories are in a position to work out methods for utilising the great masses of produce which come in bountiful seasons and which under present conditions are simply wasted. Thousands of tons of fruit, vegetables and potatoes are left on the farms to rot almost every year. For the villagers the factory affords the prospect of employment.

So far we have been dealing with the moderate sized farms run by men farming 100 to 400 acres, who constitute the backbone of British agriculture. The other two groups—the larger ones and the smaller farms—have a special interest because of the very important social problems associated with them. To begin with the large ones, many of these offer the possibility of intensive mechanisation. Large scale operations backed up by machinery and labour saving devices always suggest efficiency and economy. For some branches of agriculture, particularly corn production,

large scale machine work is much cheaper than the ordinary production by horse labour paid at the customary rates. But the system has its dangers. In the summer of 1933 I visited the Western Canadian prairies and some of the French Canadian settlements. The prairie wheat production is highly efficient, labour is cut down to a minimum, on the best managed farms one man suffices to one square mile, and some of the older homesteads are no longer needed so that they are falling out of use. But with the best efficiency of working the costs cannot be brought down below a definite point. The tractor will suffer no curtailment of its ration of oil and paraffin, the mechanic will not take a cent less than the predetermined rate of pay; bank, mortgage and elevator charges have to be met. So long as the price of wheat remains above this fixed minimum all goes well, but as soon as it falls a few cents a bushel below, the system breaks down. I saw men gallantly trying to meet the situation by giving up the tractor and using horses, which are much more elastic in their requirements, needing neither oil, paraffin nor mechanics, and at a pinch capable of finding their own food on the prairie. But it was all unstable, and the prairie towns were in a bad way. Of course they will pick up again immediately the price of wheat rises above the necessary minimum, but in the meantime they are suffering loss.

The French Canadians, on the other hand, are in a sounder position. They do not go in for this intense mechanisation. Theirs is essentially peasant proprietorship, the whole family can work. They produce primarily for themselves, and the brightness and healthiness of the children and of

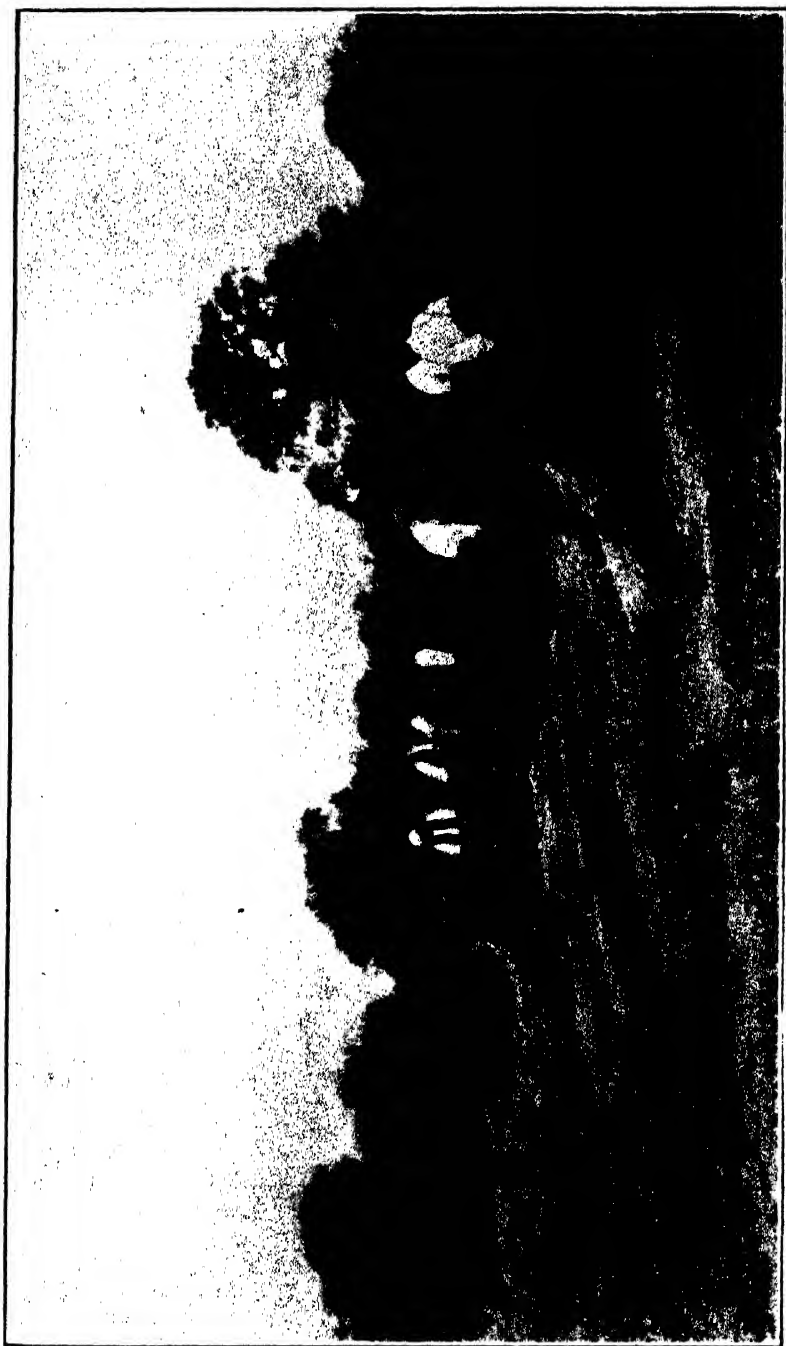


PLATE I.—The old way : Harvesting wheat at Rothamsted about 1880.

THE FUTURE OF BRITISH AGRICULTURE

the grown-ups show that they live well. The communities are self contained, centring round the Church, with quite a cheerful social life. They sell their excess produce and naturally hope for high prices, but if low prices come they can always eat the food themselves. Their output per man is less than on the mechanised farms, but per acre it is more, and they have no fixed expenses like the machinery farmer ; the bullock costs very little to maintain, and when past work he still has value, for he can be eaten, while the tractor is a perfectly useless eyesore when the farmer has done with it. One lady showed me her farm account book : it was quite simple and had only receipts entries, I asked to see the payments entries, but was merely told there weren't any !

We are not likely to see the contrast between the machine and the peasant presented in quite so striking a fashion in this country, because none of our farmers have quite the same view of land and of life as the French Canadians, though perhaps the Welsh come near ; nor are any of our leading farmers quite as specialised as the prairie grain farmers. But this mechanised farming has appeared on the Downs in the South of England and in the Eastern Counties ; workers have become unnecessary and their cottages have fallen into ruin. Fortunately, however, the best of the English "mechanisers" realise the dangers of extreme specialisation, and they are combining other agricultural activities with grain production. Several experiments are being tried. Pigs and poultry fit in very well ; they economically consume any grain not quite up to market standards. After two or three years of corn, grown cheaply with big machinery, two years of grass can be

taken for the pigs, poultry and other animals, then can follow some such crop as sugar beet or kale. Mr. Dudley is working somewhat on these lines on his chalk farm in Hampshire. Market garden crops lend themselves to this type of large mechanised treatment and they require numbers of men per acre. Mr. Secrett's farms in Surrey, Sir Bernard Greenwell's in Suffolk, Mr. Bomford's in Gloucestershire are all examples. Much ingenuity is expended in utilising the waste products of industry. Motor cars unfit for the roads are bought at low prices and used for a multitude of purposes on the farm. Rubber-tyred wheels are put on to farm carts, thereby increasing the efficiency of the cart by some 50 per cent; the friction is so much less that the horses can move more quickly and the cart can be more heavily loaded; also the rubber wheels do not cut up the farm roads so badly as iron wheels do. One particularly ingenious farmer got over the cost of erecting silos by using dismantled steamer funnels. Electrical appliances promise to be exceedingly helpful, while crop-drying machines open up the possibility of saving much corn, and of establishing central crop-drying factories supplied with raw material by farmers working on contracts. By a highly intelligent use of mechanical devices Mr. Hosier produces milk on land in Wiltshire which previously had been far less useful; his system is to keep the cows out all the time, to milk them out of doors in movable sheds but to arrange the operations so that the land is systematically manured and so enriched by the animals. Some of this mechanisation has the effect of keeping in cultivation land that would otherwise be in danger of becoming derelict.



PLATE II.—The new way : Modern Combine Harvester on Mr. R. Dudley's Mechanised Farm 1934.

THE FUTURE OF BRITISH AGRICULTURE

Mechanisation has the further advantage that it enables the farmer to obviate some of the difficulties of our climate. Many seasons are bad simply because the fine weather at sowing and at harvest did not last long enough to allow the work to be

TABLE 5.
NUMBERS OF SMALL HOLDINGS.
ENGLAND AND WALES.
THOUSANDS.

Acreage	1919	1924	1931	1934
1-5	81.2	76.9	71.2	68.5
5-20	113.4	111.9	102.3	98.8
20-50	78.0	79.5	77.4	76.0
TOTAL ...	272.6	268.3	250.9	243.4

NUMBER OF NEW SMALL HOLDINGS ESTABLISHED.
ENGLAND AND WALES.

	1919-24	1926-30	1931-33	Total
By County and County Borough Councils ...	16,550	693	670	17,913
By the Ministry	512*	116†	—	628*
Total established	16,833	809	670	18,312
Net change in number since 1919	-4,238	-13,401	-7,573	-25,212

* Including 229 on estates transferred to County Councils and therefore presumably included in the line above.

† Under the Small Holdings (Colonies) Acts.

done. The machine overcomes this by speeding things up very rapidly. Used as these particular farmers are using it, mechanisation need not deplete the countryside: if it did it would be open to serious criticism in a crowded country like England.

At the other end of the scale come the small holders who present the most difficult problem of all. Vast efforts have been made for many years to establish them on the land; some have succeeded, some have failed. Since 1919 the total number of small holdings has decreased, many that were started with great enthusiasm failed to survive.

Yet I believe that small holdings in some form or other must always play a part in our farming and our social life. There is a stability about the French peasantry and the French Canadian farmer that is worth a great effort to achieve. The difficulty, of course, is the marketing of the produce, arising out of the fact that production per man is less efficient than on the large farm, and the small holder being in a small way cannot be sure of fulfilling his contracts.

The most-successful small holdings are those of Denmark. They are specialised and organised to produce three commodities only, eggs, bacon and butter, to supply the English breakfast table; but they are also co-operative, and the organisation is so complete that the Danish combinations can undertake to fulfil even the largest contracts without the slightest fear. The small farmers are able to devote themselves entirely to production, and having only three things to produce they become highly expert. They have nothing to do with working up the produce, they simply deliver

THE FUTURE OF BRITISH AGRICULTURE

it to the factory where the butter and bacon are made, the eggs graded, and the final products sold in bulk with the minimum of expense and the maximum of profit.

Efforts have been made to establish such colonies here, but in the main they have failed. Two essential requisites are continuity and leadership for work, always very difficult to secure for small enterprises, as good men are quickly caught for big ones. Perhaps the new canning factories or the poultry marketing scheme will supply this need. But a third essential requisite is more difficult to secure—an assured market.

There is, however, a second type of settlement possible, fundamentally different, as production is not specialised but general. The main purpose is to live and not to sell, the community is self-contained and self-sufficing, each member produces what he can and exchanges his excess over his own requirements for things he does not produce. Several experiments in this direction are being made in this country; the best known is that of Prof. Scott, in which the medium of exchange is a token and not currency. The system is not unlike what in practice often happens in the French Canadian settlements. It obviously requires considerable organisation and leadership. In French Canada this is supplied by the Catholic Church, the central power in the village. Other successful examples can be found in the British Empire and in the United States, especially in the earlier days, but the settlers have always been united by some common spiritual bond, always far more potent than the bond of common financial interest. Christchurch, New Zealand, one of the most attractive places in the Empire, was founded

by a group of Church of England settlers who from the outset cared at least as much for their Church and their newly founded College as for their markets.

Settlements of this type could be made very attractive. All kinds of small machines and appliances are now available to mitigate the severity of farm work, and a group of farmers and craftsmen producing for themselves could build up homes to their liking, free from the cares and anxieties of business life.

These social problems must be kept distinct from ordinary agriculture. Our agriculture, however, should not be regarded as a purely economic enterprise but as the counterpart to our town life and as a means of solving some of the problems arising out of our extreme industrialisation. Smallholders' settlements could be made to contribute a good deal to our social peace and to give to many a new hope in life. They are not likely to do much to feed the towns, however. The nation's home-grown food will always, I think, come chiefly from the moderate and large sized farms. Remembering too that the Englishman is at heart a countryman, we may hope that there will always be men of means able and willing to take up large scale farming and introduce new methods, or effect improvements in cultivation and live stock. British agriculture owes a great deal to men who after amassing wealth in the cities retired to the country to foster agriculture and agricultural science. As a countryman, I always think that such men are following the advice recorded by Plato : " Having acquired wealth, begin to practice virtue."

[E. J. R.]

THE CLIMATIC AND SOIL REQUIREMENTS OF TEA

HAROLD H. MANN¹

(*Rothamsted Experimental Station*)

THE distribution of tea cultivation in the world is at first sight peculiar. On the one hand, the crop seems not at all precise in its demands in the matter of climate, for it is found from comparatively far north, where frost is common and more or less temperate conditions prevail, to the immediate neighbourhood of the equator, where it is cultivated on a very large scale. On the other hand, its cultivation is curiously restricted; for, with the exception of recent plantings in Africa and Russian Georgia, it is hardly found anywhere as a commercial crop save in the south-east of Asia and the islands adjoining this region. There is no commercial cultivation as yet on the American continent (though there seem to be possibilities of development in Brazil), or in south Europe; and in Africa it has so far been grown only in the eastern half of the continent.

Tea growing is limited by labour considerations as well as by those of climate and soil; it demands more hand-labour per acre than almost any other tropical cultivation, and this hand-labour must be cheap if the cultivation is to be profitable. Such cheap labour is available in precisely those portions of the world where tea cultivation has become established. Despite this fact, it is at least curious that the distribution of one of the most profitable of tropical and semi-tropical crops should be so limited.

One reason for the present distribution lies in the centre from which the plant has spread. The original home of the tea plant is believed to be the great mountain area of Further India, between India, Burma, and China. In the mountain valleys of this area there are still whole districts where tea trees abound in the forest growths. From this centre, the plant appears to have spread to China, and, more slowly, it became commonly used by the hill peoples of the Shan states, the Naga and other adjoining hills, and, to a certain extent, in the Irrawaddy and Brahmaputra valleys. The principal spread was, however, to the north, and, in China, tea became at a very early date one of the characteristic cultivations of the country. From this centre in China, the prepared product was distributed in varying degrees to all parts of the civilized world. Up to the 'thirties of the last century, the production of tea on a commercial scale was exclusively carried on in China, and, in fact, it was believed by many that a drinkable tea could only be produced in that country. Between 1830 and 1840, however, tea was cultivated experimentally in several parts of India and Java. Some of these experiments succeeded far beyond the hopes of the pioneers; others failed completely; whilst in other areas tea growing has maintained a feeble existence and has not expanded. The centres of greatest success were Assam and, in fact, all the north-east corner of India, to a less extent the Nilgiri mountains in south India, at a later date Ceylon and Travancore, and finally

¹ Formerly Scientific Officer to the Indian Tea Association, now Assistant Director, Woburn Experimental Station.

Java and Sumatra in the East Indies. The areas where tea is still grown, but where the cultivation has languished, may well be represented by the Himalayan tea-districts of India, which lie to the west of Darjeeling, such as Kumaon, Kangra, and Dehra Dun. Outside India, Natal is in a similar position. In America, as already stated, there is at present no commercial tea cultivation of any importance. In Russia, where tea has been grown for only thirty to forty years, the cultivation has but recently been extended, and it may still be regarded as experimental. In Africa, a flourishing though small tea industry exists in Nyasaland, and a similar small industry is becoming established in Kenya.

In certain directions the tea plant may be said to be very tolerant of considerable variations in climate; in others it is most precise in its demands. The conditions which are found in all areas where tea cultivation is an established success are detailed below, and the suitability of an area can be determined by seeing how far the conditions in that area approach these ideal requirements:

(1) There should be rarely a month during the year when *some* rain does not fall, and as a result, the soil should never become dry for more than a very short distance below the surface.

(2) The total rainfall during the year should exceed 60 in.; in the best areas it is considerably higher. At greater elevations and in more temperate conditions, less rainfall is required than where really tropical conditions prevail.

(3) The minimum temperature of the year should never fall below freezing-point, and if it does, such occurrences should happen infrequently and at night only. This demand is not absolute, but if the winter temperature is more severe than that specified, only the northern or China varieties can be cultivated successfully.

(5) The shade temperature at the hottest time of the year should not much exceed 90° F., and when high temperatures occur the relative humidity of the atmosphere should also be high. The conjunction of a high temperature and a dry atmosphere at any time of the year is very dangerous, and its occurrence very substantially limits the extension of successful tea cultivation.

(6) The daily range of temperature should be small, even in the cold weather, and particularly small during the season when growth is most vigorous.

(7) The absence of strong, and especially of strong and *dry*, winds at any time of the year is important.

Naturally, these points are not all of equal moment, and the absence of some of them can be made good by changes in the method of cultivation; but it will be seen that we are dealing with a crop whose range is necessarily limited.

For successful cultivation, tea has, as shown above, certain well-defined requirements in regard to climate. Similar restrictive conditions exist in regard to soils. There are, in point of fact, certain characteristics of tea soils, relating to both physical condition and chemical composition, which seem to be absolutely essential if really satisfactory and permanent tea cultivations are to thrive. If these essential conditions

are satisfied, then tea will grow well in varied types of soil; if not, the apparently most favourable situations will spell failure.

Tea is grown on alluvial or sedentary soils. Most of the important tea estates in north-east India lie on practically flat alluvial land, some of rather old types and the remainder of quite recent deposition. The tea of Ceylon and also of south India is grown on sedentary soils that are mainly derived from gneiss and granite. The same is the case in Sumatra. In Java, the chief tea areas possess soils which lie on volcanic rocks, and on volcanic rocks of rather varying types. In Africa, tea is grown successfully in Nyasaland on alluvial soils that were directly washed from a great gneissic *massif*, and equally well on sedentary gneissic soils. Further, tea thrives well on red soils, which are most frequent in Ceylon, south India, and on the older alluvial areas of Assam, the Duars, and other parts of north India. It is equally successful on many purely grey soils in close proximity to the red soils. The largest annual yields of tea have probably been obtained from tea planted on well-drained peat several feet deep in Cachar and Sylhet.

The special soil characteristics that are really essential for successful tea cultivation may now be discussed. The first of these is a deep soil, and the depth should be greater as the liability to drought increases. Such a deep soil is not of great value unless the tea bushes can force their roots well into the subsoil. There seems little doubt that the capacity of a tea bush to penetrate a hard or sticky subsoil is limited, and very few perennial plants have so little power of penetrating an unsuitable layer of subsoil—whether this unsuitability is caused by hardness, stiffness, or lack of drainage—as has the tea bush. This does not mean that tea can never be grown on soils with a subsoil into which tea roots cannot easily penetrate, for many very successful tea estates exist where the subsoil inhibits the formation of deep roots either of tea or of any other plant growing on them. However, such tea estates in actual practice are very liable to drought; they depend for their success on the great richness of the surface soil, and are liable to very rapid deterioration. Apart from such exceptional cases, it may be said that soils likely to be suitable for tea should be deep with the lower layers porous, well-drained, and easily penetrable by tea roots. It is possible that hard subsoils may be opened by the use of shade trees planted amongst the tea, and the value of such trees in assisting the penetration of tea roots to considerable depths is gradually being recognized.

The normal development of the roots of the tea plant under conditions of ordinary cultivation [1] may be said usually to consist of a deep tap-root or deep secondary roots, whose primary purpose appears to be to absorb the water that the plant needs, and a series of sub-surface roots, which are normally found from 4 to 6 in. below the surface of the soil, if the tea bush is properly planted. To obtain this type of root-development, it is clear that only soils in which the ordinary forest or jungle growth is found to penetrate deeply into the subsoil should be selected. If the roots of jungle trees do not penetrate deeply, it is necessary to examine whether there is any sign of waterlogging of the subsoil either from springs in the ground or from other causes. If there is the least sign of the

subsoil being waterlogged at any time of the year, it will be necessary, if successful tea cultivation is to be carried on, to arrange for thorough drainage of the land to a depth of at least 3 ft., for the purpose not only of taking away the surface water, but also of removing the tendency of water to accumulate in the subsoil. The importance of the easy penetrability of the subsoil must be insisted upon, because there have been, all over the world, very frequent disappointments from planting tea where the subsoil conditions have either not been naturally satisfactory or have not been made so before the planting was begun.

From the chemical point of view, good tea soils must fulfil the following two requirements: (1) the soil should not contain more than a trace of lime, and (2) the soil must be definitely acid.

The presence of more than very small amounts of lime in the soil, at least in the form of carbonate of lime, seems almost fatal to the tea plant. There are cases on record in which lime is said to have been used successfully as a manure in tea gardens, but such records are very few, and in the vast majority of cases the addition of lime—which has so frequently been recommended by chemists after analysing the soils—has either had no effect at all or it has been actually injurious to the crop. In a recent exhaustive examination of the results of applying lime to healthy tea on a fairly acid soil in Assam, Carpenter, Cooper, and Harler [2] showed that in no case was there any benefit from the use of lime, although the available lime (i.e. the amount extractable by 1 per cent. citric acid) was less than 0.05 per cent. They claim, but without giving the evidence, that lime has proved useful when peaty material has been used as a tea manure and the tea has subsequently deteriorated. The latest evidence is that of Prillwitz [3], who states (1932) that in no case, in all his experiments, has manuring with lime had any favourable effect on the growth of the plants. On the other hand, I have myself recorded [4] successful tea where the lime-content of the soil, as extracted by hydrochloric acid, was above 0.5 per cent., but this, I believe, is the maximum ever recorded. The average amount of lime extractable by hydrochloric acid in good tea soils, in the main tea districts of north-east India, does not exceed 0.12 per cent. Further, if in any area where tea is being planted there happens to be a patch of soil where lime occurs in more than a very small percentage, the tea growing, if it grows at all, upon such a patch of soil is invariably very inferior. In this case, it is not yet clear whether it is the presence of more than a trace of lime that is the evil influence, or whether it is chiefly an indicator of too little acidity for tea. The fact that the tea plant is definitely a *calcifuge* must never be forgotten in selecting areas for tea cultivation.

The other special need of a tea soil is that it should have an acid reaction. It is unlikely that tea cultivation will be a commercial success in any soil which has a pH value exceeding 6.0, although Eden [5] has recently recorded some good tea soils in Ceylon with a pH value slightly higher than this; in one case going up to pH 7.3. Such cases are so exceptional, however, that they need a much closer examination than has been given to them before they can be accepted without reserve.

The ideal pH value for a tea soil probably lies somewhere between

5.2 and 5.6, though many successful tea soils have a much lower value than this. One case is recorded by Carpenter in which good tea was growing in soil of pH value 3.6. The scientific workers of the Indian Tea Association in Assam consider that great importance should be assigned to the difference between the pH value of the soil in water solution and that obtained in a normal solution of potassium nitrate. The latter always, of course, tends to give the higher acidity, and the difference between the two values is termed the 'reserve acidity'. If this is large, the soil is likely to be a good tea soil, provided the original pH value in water solution is within the suitable limits; if the difference is small, there is less certainty [6].

The effects on the tea plant of soil that is either definitely alkaline or insufficiently acid have been indicated by Eden in a recent publication [7]. He contends that soil alkalinity leads to failure of the main axis of the plant to elongate, to the leaf-buds being crowded together, and to the falling-off of the leaves at an early stage, leaving many scars close together. Often only leaves near the growing-point are left and side-shoots are absent. The effect on the roots is stated to be equally remarkable. In bad cases, there are no tap-roots, the appearance being as if these had been bitten off. Lateral roots are few and concentrated at the bitten-off points. There are few root-hairs and these are discoloured and brittle.

The whole question of the acidity of tea soils is so important that it would appear that the first test to be made on any soil intended for tea cultivation should be the determination of the pH values of the soil and subsoil. This matter is of peculiar interest in the case of tea, as no other crop seems to be so precise in its demands. The importance of the matter may be illustrated by the fact that the existence, for any length of time, of a native hut on a piece of ground, especially if cattle have been kept round the hut, will usually cause the acidity to decrease to such an extent as to make the growth of successful tea almost impossible on such areas.

We have thus, as definite requirements for tea cultivation, a surface soil normally rich, containing only traces of lime—at least in the form of calcium carbonate—and possessing a definitely acid reaction. Such a soil should have a subsoil that is porous, easily penetrable, and well-drained. If these requirements are met, and the climate is suitable, tea will grow, and grow well, though of course the vigour of growth will depend on the richness in ordinary plant-food of the surface soil, say to a depth of 8 or 10 in.

So far as the requirements of ordinary plant-foods are concerned, it has long been recognized that the vigour of tea depends largely on the abundance of available nitrogen, an abundance which is usually secured, at any rate in virgin tea soils, by the presence of large quantities of organic matter. A large excess of nitrogen, either in organic or other forms, seems, however, to be disastrous to the quality of the tea produced. Cooper in a recent paper [8] states that on the soils with which he was working in Assam he found he could add each year 40 lb. of available nitrogen and no other fertilizers without reducing quality, but if more nitrogen were used, then phosphoric acid and potash would have to be

applied if quality was not to suffer. It seems clear, in fact, that if the content of organic matter in soils used for tea exceeds a certain limit, and is thus able to provide more nitrogen than corresponds to the phosphoric acid and potash available, then the quality of the tea produced will decline. This happens, for instance, when tea is grown on peat soils, on which the yield is very high, but despite every effort to pluck and manufacture properly, nobody has succeeded in producing anything better than a common grade of tea. The same appears to be true to a less extent in growing tea at high elevations near the equator. Here the soil is nearly always initially very well provided with organic matter, and hence with a larger proportion of nitrogen relative to the other manurial constituents. As a result, the quality of the tea produced under such conditions during the first years of an estate is generally lower than that which can be obtained later on.

Apart from this point, the relationship of the soil on which tea is grown to the quality of the product is still very little understood. So long ago as 1907, I suggested that my analyses of the tea soils of north-east India indicated that there was a close correlation between the amount of phosphoric acid in the non-silicate portions of the soil and the quality of tea likely to be produced. Though the areas indicated at that time as likely to be capable of giving improved quality have since justified the prediction, it cannot yet be stated that there is any certainty as to a real connexion between the phosphoric acid in the soil and the quality of the tea produced. Recent opinions have been expressed by Cooper [9] that the presence of this constituent has no effect on quality, and by Carpenter [10] that phosphoric acid 'tends to steady up the flushing and in that manner makes for slower growth and better tea'. He also says, however, that it is the general opinion that much of this plant-food makes for stalky teas. The whole question of the influence of the phosphoric-acid content of the soil on the quality of tea must be regarded for the present as uncertain, and it should be the subject of further research.

The influence of available potash in the soil on the healthiness of the tea bush and on the quality of tea has also been the subject both of speculation and of experiment. As a result, it seems to have been found that potash in abundance tends to keep the tea bushes free from fungus attack, and to prolong the growing period, so that the bushes provide a supply of leaf later in the season than would otherwise be the case [11]. The importance of abundant potash in tea soils, as a means of resisting the greatest insect pest of tea, the so-called 'mosquito bug' (*Helopeltis* sp.), has been stressed by Andrews [12], who regards the ratio of available potash to available phosphoric acid as a significant factor in determining the liability of an area of tea to attack by this pest. Tunstall has suggested that abundant potash in the soil leads to storage of starchy material in the frame of the tea bush at the end of the season, and that this reserve of starch tends to give a more healthy growth early in the following season when the bushes are recovering from pruning. On the other hand, in Ceylon the importance of excess of available potash is not considered to be great, and Eden has reported recent experiments indicating no response to potash manuring [13].

The whole question of the importance of a large amount of available potash in the soil must still be considered as in a state of flux, though the indications are that it may at least play a part in the maintenance of a healthy bush.

Other constituents of the soil have from time to time been credited with special importance for the healthy growth of the tea bush or for the quality of the tea produced. Thus Bamber [14] considered that there was a clear relationship between the amount of the lower oxide of iron in tea soils and the quality of tea. Further investigation has, however, failed to confirm his views, and there is insufficient evidence to suggest that importance should be attached to this constituent of tea soils.

Again, repeated suggestions have been made that the presence in the soil of assimilable manganese has something to do with the high quality of tea from certain districts, and Bamber seemed to think that addition of manganese to the soil caused infusions made from the leaf produced to have a brighter appearance. In the early days of the investigation of tea soils, Nanninga [15] showed that the effect of manganese, when present in varying quantities in the soil, is most marked on the composition of the tea leaf. However, this matter has not really been followed up, and much more experimental work is required before it can be accepted that tea quality should be connected with the available amount of manganese in the soil.

The possibility of magnesia starvation has been referred to in connexion with tea soils in Nyasaland [16], where the deficiency of magnesia in tobacco soils seems to have been proved, but at present the evidence is insufficient to support the view that such deficiency is anything but a very rare phenomenon.

On the other hand, recent work seems to show that a deficiency of sulphur may be the cause of tea not flourishing in many areas and may give rise to a specific affection of tea known in Nyasaland as 'tea yellows'. This disease, which has been investigated recently in Africa by Storey and Leach [17], and may be more widespread than has been generally recognized, seems to be definitely caused by lack of sulphur in the soil. It can be rapidly cured either by the use of sulphur itself or by manuring with sulphates, whether in the form of sulphate of ammonia, sulphate of potash, or even sulphate of magnesia.

This survey of our present knowledge of certain aspects of tea soils in the principal tea-growing areas of the world shows, if nothing else, how fragmentary is our real knowledge of the requirements of tea soils for giving large yields, for producing healthy growth, or for giving a high-quality product. Experimental stations now exist in most of the great tea-growing areas of the world, and these should considerably add to our knowledge in the near future. The matter is obviously of great importance to the future of what is one of the most extensive tropical and semi-tropical cultivations of the world, for all the work hitherto done in connexion with tea soils still leaves us with a totally inadequate knowledge of the relationships of the soil conditions to, at least, the quality of tea likely to be produced, and to the healthiness of the bushes which produce the tea.

REFERENCES

1. A. C. TUNSTALL, Indian Tea Association, Bull. No. 1, 1916.
2. P. H. CARPENTER, H. R. COOPER, and C. R. HARLER, Quarterly Journal of the Scientific Dept. of the Indian Tea Association, 1925, Pt. 1, pp. 1-11.
3. P. M. H. H. PRILLWITZ, Archief voor de Thee-cultuur in Nederlandsch-Indië, 1932, No. 2.
4. H. H. MANN, Tea Soils of North-East India, Indian Tea Assoc. Sci. Dept., Public. No. 22, 1907.
5. T. EDEN, Ann. Rep. Tea Res. Inst. Ceylon, 1928, p. 17.
6. C. J. HARRISON, Quart. J. Sci. Dept. Indian Tea Assoc., 1930, Pt. 4, p. 170 and 1932, Pt. 2, p. 70.
7. T. EDEN, Ann. Rep. Tea Res. Inst. Ceylon, 1931, p. 36.
8. H. R. COOPER, Quart. J. Sci. Dept. Indian Tea Assoc., 1932, Pt. 1, p. 36.
9. —, *ibid.*, p. 39.
10. P. H. CARPENTER, *ibid.*, 1930, Pt. 2, p. 87.
11. —, *ibid.*, 1933, Pt. 1, p. 25.
12. E. A. ANDREWS, Factors affecting the Control of the Tea Mosquito Bug, Indian Tea Assoc. Bulletin (no date).
13. T. EDEN, Ceylon Tea Quarterly, 1933, 6, 25.
14. M. K. BAMBER, Report on Ceylon Tea Soils, Colombo, 1900.
15. A. W. NANNINGA. Meded. Lands Plant., 1903, No. 65.
16. Nyasaland Dept. Agric., Bull. No. 4, 1932.
17. H. H. STOREY and R. LEACH, Ann. Appl. Biology, 1933, 20, 23.

(Received July 24, 1933)

THE INFLUENCE OF SEASON AND OF THE APPLICATION OF LIME ON THE BOTANICAL COMPOSITION OF GRASSLAND HERBAGE

By WINIFRED E. BRENCHLEY, D.Sc.

(From the Botanical Department, Rothamsted Experimental
Station, Harpenden, Herts.)

(With 5 Text-figures.)

CONTENTS.

	PAGE
Introduction	183
A. Changes in herbage due to season	185
(1) Seasonal variation in the proportion of grasses, leguminous and miscellaneous species	186
(2) Seasonal variation in the proportion of individual species	189
B. Rate of change due to liming	198
(1) Response of main groups of grasses, leguminous and miscel- laneous plants to liming	198
(2) Response of individual species to liming	199
Summary	206
References	207

INTRODUCTION.

IN the year 1856 John Bennett Lawes established the classical Park Grass Plots at Rothamsted to determine the effect of fertilisers on the yield and botanical composition of hay. An area of long-established grassland was divided into strips dressed with various combinations of manures, chiefly artificial, and with certain modifications the same treatment has been continued annually till the present day. In 1903 a system of periodic liming every four years was instituted on most of the plots, and in 1920 the remainder were brought into line. During the period 1856-1919 the changes in the herbage were recorded by frequent analyses of hay samples into the three main groups of grasses, leguminous and miscellaneous plants, while on seven occasions complete separations into individual species were made and the results published in detail (1, 2). By 1919 consideration of all the results focused attention on two further problems:

(1) Given constant treatment, how far is the botanical composition of the herbage influenced by seasonal variations from year to year?

184 *Influence of Season on Botanical Composition of Grassland*

(2) How quickly is the botanical composition of grass cut for hay affected by change of manurial treatment, apart from seasonal variation?

In 1920, various plots were selected for complete botanical analysis over a period of years.¹ These included three representative plots of which the unlimed halves had had no change of treatment since 1856, and had received no manure, mineral fertilisers only, and minerals and sulphate of ammonia respectively, together with all those plots which first came into the liming system in 1920. Certain other analyses were also made for special purposes dealt with later. This provided material from a group of plots in which the herbage might be considered to have reached a position of equilibrium as far as direct manurial influence is concerned, and from another group in which the botanical composition passed through a stage of flux owing to the change of treatment. In some plots of the second group the complete analyses were only carried out in alternate years, while in the first they were made annually for at least five years. Partial separations into groups have been made on the same plots where the time factor precluded further complete analyses, and these have been continued until the present day.

The history of the plots considered is here tabulated for reference to avoid undue repetition later.

GROUP 1. *Plots with continuous manurial history.*

Sampled yearly.

Plot 3, U. and L.	Unmanured.
Plot 7, U. and L.	Complete mineral manure: super; sulphates of potash, soda, and magnesia.
Plot 9, U. and L.	Complete mineral manure and sulphate of ammonia (= 86 lb. N).

GROUP 2. *Plots with change in manurial treatment and/or later liming.*

Sampled yearly.

Plot 14, U. and L.	Complete mineral manure and nitrate of soda (= 86 lb. N).
Plot 18, U., L.L. and H.L.	Mineral manure (without super), and sulphate of ammonia (= 86 lb. N), 1905 and since, following minerals and ammonium salts supplying the constituents of 1 ton hay, 1865-1904.
Plot 19, U., L.L. and H.L.	Farmyard dung in 1905 and every fourth year since (omitted in 1917), following nitrate of soda (= 43 lb. N) and minerals, 1872-1904.
Plot 20, U., L.L. and H.L.	Farmyard dung in 1905 and every fourth year since (omitted in 1917); each intervening year plot 20 receives sulphate of potash, superphosphate and nitrate of soda (= 26 lb. N), following nitrate of potash and superphosphate, 1872-1904.

¹ It is hoped to publish the complete analytical data in the *Report* for 1934 of the Rothamsted Experimental Station. In the present paper skeleton tables only are given of a few species to illustrate salient points.

Sampled in alternate years.

Plot 5 ¹ , U.	Unmanured, following ammonium salts (=86 lb. N), 1856-97.
Plot 5 ² , U.	Superphosphate, and sulphate of potash, following ammonium salts (=86 lb. N), 1856-97.
Plot 15, U. and L.	Complete minerals as plot 7, following nitrate of soda (=86 lb. N), 1858-75.
Plot 17, U. and L.	Nitrate of soda (=43 lb. N).

U. = unlimed; L. = limed 1920 and every four years after;
 L.L. = light limed; H.L. = heavy limed.

A. CHANGES IN HERBAGE DUE TO SEASON.

The influence of season on the constitution of the herbage is reflected in the relative proportions of the main groups of grasses, leguminous and miscellaneous species, and in the variation in the proportions of the individual species within these groups.

The analytical data available is somewhat unwieldy, and for the sake of simplicity attention will be concentrated on a group of plots giving a representative range of manuring, *i.e.* no manure, and mineral fertilisers with and without nitrogen applied as sulphate of ammonia and nitrate of soda respectively (plots 3, 7, 9 and 14). In the first three cases the limed areas can also be considered, as the influence of lime had become stabilised since its first application in 1903. Pertinent instances from other appropriate plots will be cited where applicable.

It is impossible to give the fifteen years' meteorological data in full, but some idea of the range of seasonal variation may be gained from Table I, which gives the essential figures on a three-months basis.

Table I.
Meteorological data, 1919-33.

	Rainfall (total) in.				Bright sunshine (total) hours				Temperature (mean) ° F.			
	Jan. to Mar.	Apr. to June	July to Sept.	Oct. to Dec.	Jan. to Mar.	Apr. to June	July to Sept.	Oct. to Dec.	Jan. to Mar.	Apr. to June	July to Sept.	Oct. to Dec.
1919	11.3	5.4	7.5	8.9	188	609	507	218	35.9	52.0	57.5	40.5
1920	5.2	7.8	8.3	5.9	277	565	409	254	41.9	52.3	56.2	43.5
1921	3.7	3.2	4.0	5.1	253	641	559	270	42.7	52.1	61.4	45.3
1922	7.9	6.1	10.4	5.3	272	659	379	252	39.1	51.3	55.4	42.7
1923	7.9	3.8	8.7	9.6	189	398	670	245	41.5	49.4	59.4	41.1
1924	4.8	9.8	10.5	11.5	287	548	524	158	38.0	51.9	57.8	45.4
1925	7.2	4.3	10.7	7.4	210	604	441	251	40.0	51.8	57.9	41.8
1926	6.2	7.9	5.8	8.5	206	443	480	208	41.8	51.2	60.3	42.3
1927	8.8	6.6	12.9	8.2	234	576	420	184	40.5	50.9	57.8	41.8
1928	8.6	4.6	5.5	9.8	258	527	681	248	41.1	50.5	58.9	43.9
1929	2.6	5.7	2.3	17.1	291	643	646	273	35.6	50.2	61.2	44.5
1930	5.6	6.2	8.5	9.2	231	524	546	243	39.6	52.1	58.7	43.7
1931	4.5	7.5	9.5	5.0	284	486	434	228	38.0	51.7	56.4	44.2
1932	4.4	7.5	6.7	7.3	262	485	441	208	39.0	50.3	60.0	43.7
1933	7.1	3.5	4.6	3.0	370	562	673	187	39.4	53.3	62.7	41.3

186 *Influence of Season on Botanical Composition of Grassland*

(1) *Seasonal variation in the proportion of grasses, leguminous and miscellaneous species.*

With complete dressings of minerals and nitrogen (plots 9 and 14) the proportion of grasses is very high, sometimes reaching 99 or 100 per cent., and as a general rule is little influenced by season. Occasionally the grasses are somewhat depressed by an abnormal development of some miscellaneous species, as *Rumex acetosa* on plot 9 in 1919, and *Anthriscus sylvestris* on plot 14 in 1924 and 1925. In no instance has the almost negligible proportion of leguminous plants been significantly increased.

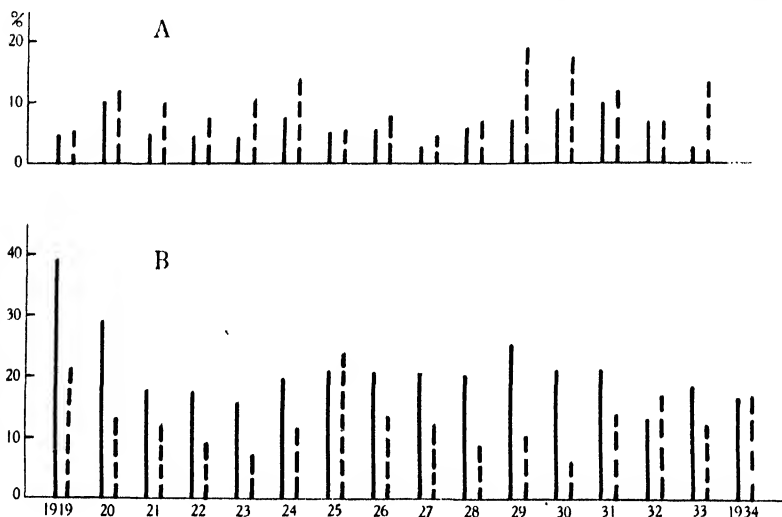


Fig. 1. A. Percentage of leguminous plants on plot 3 (unmanured), 1919-33. B. Percentage of miscellaneous species on plot 7 (minerals), 1919-34. ——— Unlimed. - - - - - Limed.

The omission of phosphate from an otherwise complete fertiliser (plot 18) has prevented the grasses from becoming so entirely predominant. *Rumex acetosa* has maintained its position, and in some seasons, as 1922, 1926 and 1930, has constituted about 20 per cent. of the herbage. This affords an interesting quantitative corroboration of various observations hitherto made that *Rumex acetosa* is more encouraged by deficiency of phosphate than by acidity of soil.

On unmanured plots and in the absence of nitrogen wide seasonal fluctuations occur. Without either manure or lime (plots 3 and 5¹) an increase in grasses is usually accompanied by a decrease in miscellaneous plants, the variation in leguminous species being comparatively small.

With minerals the grasses and Leguminosae vary in correlation, the miscellaneous plants remaining much more constant. Where, however, minerals have followed several years of nitrogenous manuring (plots 5² and 15) the seasonal variations are distributed between all three groups. Liming tends to exaggerate the range of variation, upsetting the relative constancy of the leguminous plants on the unmanured and the miscellaneous species on mineral plots (Fig. 1).

The use of farmyard manure with or without artificials seems to introduce a certain rhythm into the seasonal fluctuations, which tend

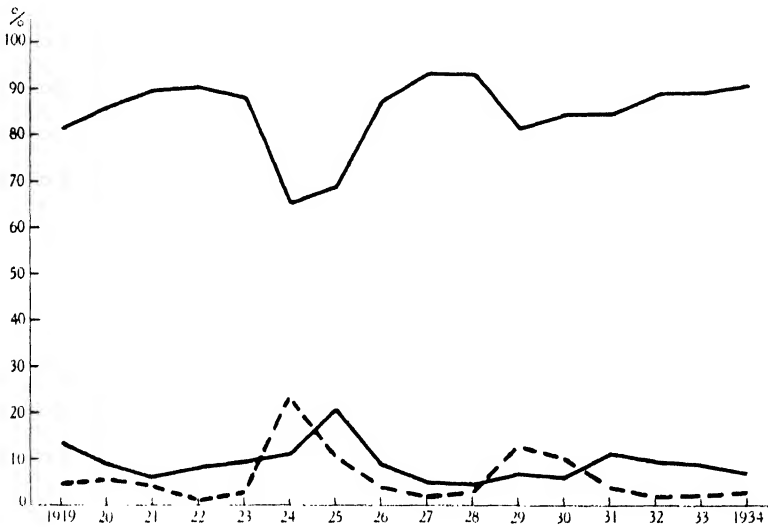


Fig. 2. Curve showing rhythmic change of proportion of the three main groups of species on plot 20, unlimed (dung and artificials), 1919-34. — (upper) grasses. — (lower) miscellaneous. - - - Leguminous species.

to increase to a maximum and then decrease to a minimum over a period of years (Fig. 2). Three of these rhythmic cycles can be traced between 1919 and 1934, the peaks of maxima and minima not always occurring in the same year in the three groups of species.

The direction of change in the proportion of the three groups year by year is not always the same with different treatments; nevertheless, in some years characterised by outstanding meteorological conditions most plots behave very similarly. For instance, in 1921 the proportion of grasses was high on most plots in a season of low rainfall, medium sunshine and comparatively high temperatures during the growing season. In 1924 the same plots gave a low percentage of grass with a

188 *Influence of Season on Botanical Composition of Grassland*

medium amount of rain, low temperatures early in the season, and much sunshine in March and April. Similar conditions gave similar results in 1929 on the unmanured and mineral plots (Fig. 3). It would seem that low temperatures early in the season tend to reduce the proportion of grasses, even though abundant sunshine occurs about April and May. On the other hand, relatively high temperatures early in the year encourage the grasses even with low rainfall and average amounts of sunshine.

The question arises as to whether the annual variations in yield have any direct influence on the botanical composition of the herbage. Comparison of the figures over a varied range of manurial treatments fails

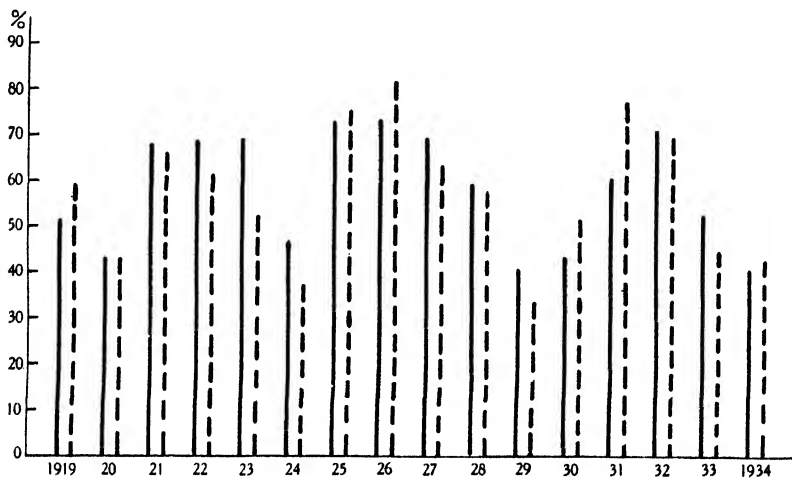


Fig. 3. Percentage of grasses on plot 7 (minerals), 1919-34.
—— Unlimed. - - - - Limed.

to reveal any correlation between the yields and relative proportions of grasses and miscellaneous plants. Though no definite association can be traced, with nitrate of soda and minerals (plot 14) the only two serious drops in the proportion of grasses and corresponding increases in miscellaneous species occurred in years of high yield; but this may be merely coincidence. With mineral manures, however, there is some suggestion of association between heavy yield and high percentage of leguminous plants, particularly on the limed areas. In 1920, 1924, 1929 and 1930 the proportion of *Lathyrus pratensis* was particularly high, ranging from 45 to 57 per cent. with lime and from 28 to 35 per cent. unlimed. In all cases except one (1929, plot 7 unlimed) the corresponding yields were high. This correlation may be connected with the trailing habit of

Lathyrus, which allows grasses and weeds to grow through it instead of crowding them out. It is also possible that the considerable extra supply of available nitrogen produced by the heavy leguminous crop encourages the growth of the other groups, thus pushing up the total yield. Apart from this, variations in yield and botanical composition seem to be entirely independent, at least as far as the three main groups are concerned.

(2) *Seasonal variation in the proportion of individual species.*

The influence of season on the relative proportion of the species in the mixed herbage of grassland presents a most complicated problem on account of the number of factors involved. Rainfall, temperature, sunshine, manuring, soil reaction and plant competition are probably but a few of the factors whose interplay determines the annual variation in the botanical composition of the herbage. In the space here available it would be an impossible task to disentangle the reasons for the seasonal changes in any detail, but an attempt will be made to correlate the major variations with the conditions in particular years.

On grassland carrying a number of diverse species growing in association, an increase in the proportion of any one species is necessarily at the expense of a decrease in one or more others. If species were all affected equally by season their relative proportion in the constitution of the herbage would remain constant. As, however, conditions that favour one plant discourage another the percentage composition of the herbage is in a continuous state of flux. Seasonal changes, therefore, resolve themselves into variations in the balance of competition from year to year. The competition between the species constituting grassland herbage is acute, but the fact that any one species is predominant under a particular set of conditions does not necessarily mean that it is in itself of necessity specially favoured by these conditions. For instance, if two species *A* and *B*, or *B* and *C* are in competition,

(a) *A* may be specially favoured by the particular conditions obtaining, while *B* is indifferent, in which case *A* will tend to predominate while *B* is reduced in quantity;

(b) *B* may be indifferent to the conditions, whereas *C* is adversely affected, in which case *B* will increase and *C* will tend to be crowded out.

This affords an explanation of the variation in the relative prevalence of certain species under different conditions of competition. *Rumex acetosa*, for instance, when grown alone gave the largest crops in soil well supplied with lime, whereas in the same experiment under conditions of

190 *Influence of Season on Botanical Composition of Grassland*

competition it was crowded out on well-limed soils, but held its own on acid soil which was less favourable to the growth of the other competing species. In any season climatic conditions are uniform over all the plots while manurial treatments and soil reaction vary considerably, whereas from year to year the meteorological conditions fluctuate widely while the soil conditions and treatment of individual plots remain practically constant. It would be surprising, therefore, if a simple response to these changes were manifested and if seasonal variations in individual species were parallel on all the plots. What actually happens is that in average seasons the proportion of any species tends to alter irregularly, rising with one treatment and falling with another. In abnormal seasons, however, the influence of treatment may be outweighed by climate, and certain species may behave more or less uniformly regardless of manuring.

Seasonal variations in the proportion of any species may either be sudden and temporary, or gradual and progressive. In the first case wide fluctuations may occur almost every year, or a species which usually shows little response to season may suddenly increase, falling back to its usual level in the succeeding year. In the second case the tendency is for slight changes to occur over a period of years to a maximum or minimum as the case may be, followed either by a reversal and a gradual change in the other direction, or by a sudden change due to an abnormal season. The latter phenomenon is then usually succeeded by a further cycle of gradual change. Both forms of response may be manifested by different species on the same plot.

On completely manured plots, where comparatively few species occur, the range of seasonal variation is large. Where sulphate of ammonia is applied (plot 9) considerable fluctuations are apt to occur even with species that are normally present in small amount, but with nitrate of soda (plot 14) these less abundant species are much more stable. This does not apply so consistently where nitrate of soda is used alone (plot 17). With no manure (plot 3) or with minerals (plot 7) the large number of species brings the relative proportions to a lower level. On the whole the major seasonal variations again occur in the more abundant species, but there are instances of relatively large fluctuations in comparatively insignificant components of the herbage.

At the risk of being categorical it is necessary to deal with each main species separately, as the responses to season and to liming are so individual that no satisfactory grouping is possible.

Agrostis vulgaris et alba (Tables II and VII). In the absence of nitrogen the seasonal variation is comparatively small and tends to run

Table II.

Percentage of grasses in Park Grass herbage.

Plot ...	3		5 ¹	5 ²	7		9	
	U.	L.	U.	U.	U.	L.	U.	L.
<i>Agrostis vulgaris et alba.</i>								
1919	8	2	4	8	5	2	12	2
1921	25	2	13	20	12	5	27	4
1922	24	4	22	15	19	5	16	4
1923	21	3	n.	n.	15	5	23	2
1924	18	3	12	16	14	1	31	3
1925	19	2	n.	n.	11	2	17	2
1926	18	2	24	21	n.	n.	25	3
<i>Alopecurus pratensis.</i>								
1919	0.3	0.6	0.7	11	2	15	0.7	26
1921	0.7	5	1	6	1	12	0.6	22
1922	3	3	5	20	1	11	14	28
1923	0.2	7	n.	n.	2	7	0.3	28
1924	4	9	0.5	6	2	10	0.5	45
1925	1	4	n.	n.	1	9	2	42
1926	2	3	0.6	13	n.	n.	0.1	24
<i>Anthoxanthum odoratum.</i>								
1919	7	3	12	5	4	0.5	5	1
1921	4	0.6	9	4	5	0.7	25	2
1922	0.9	0.2	0.5	0.4	2	0.1	8	0.4
1923	4	1	n.	n.	4	0.8	43	1
1924	4	0.3	4	0.6	1	0.2	22	0.4
1925	7	0.5	n.	n.	2	0.1	13	0.7
1926	3	0.4	12	5	n.	n.	16	1
<i>Arrhenatherum avenaceum.</i>								
1919	0.3	0.5	2	2	3	3	47	47
1921	0.3	0.2	8	3	1	5	4	43
1922	0.5	0.8	2	4	1	4	11	30
1923	0.1	0.1	n.	n.	1	3	8	35
1924	—	0.1	1	3	2	4	22	32
1925	0.2	0.4	n.	n.	5	27	20	45
1926	0.1	0.4	5	8	n.	n.	6	50
<i>Avena pubescens.</i>								
1919	4	19	0.2	2	3	9	—	—
1921	3	18	0.2	2	3	12	—	—
1922	3	11	0.3	0.7	2	5	—	—
1923	4	18	n.	n.	3	6	—	—
1924	3	16	0.3	2	1	5	—	—
1925	6	32	n.	n.	1	8	—	—
1926	4	19	0.3	2	n.	n.	—	—
<i>Dactylis glomerata.</i>								
1919	8	7	9	7	22	19	3	7
1921	12	8	14	2	14	12	1	5
1922	8	8	9	7	12	10	4	8
1923	4	3	n.	n.	10	5	0.7	2
1924	4	4	6	3	12	10	0.1	2
1925	7	6	n.	n.	34	23	0.5	4
1926	5	8	8	5	n.	n.	0.4	8

U. = unlimed; L. = limed; n. = no analysis; — = missing or below 0.1 %.

No analysis of these plots was made in 1920.

192 Influence of Season on Botanical Composition of Grassland

in the same direction over a period of years, even when the percentage present is high. Sulphate of ammonia (plots 9 and 18) induces considerable annual fluctuations, completely obliterating any regular progression of change. This does not apply to the limed part of plot 9 where the quantity is very small and stable throughout. The influence of sulphate of ammonia seems to be very persistent, for on the areas on which its application has been discontinued since 1897 the annual fluctuations still occur, though they are becoming less marked where minerals are now given (plot 5²) than on the unmanured section (plot 5¹).

No uniform parallel response of *Agrostis* occurred in any particular season, but there was a tendency towards large amounts in 1921 and 1924. With farmyard manure applied alone there has been a steady slow fall since 1921, but the addition of minerals has induced a slight rhythmic rise and fall. In neither case can a correlation with type of season be traced.

Alopecurus pratensis (Tables II and VII). *Alopecurus* usually shows little response to season even where it is present in abundance, except on the limed half of plot 9. The outstanding exception to this was in 1922 when a sudden increase in quantity occurred on many plots, even where the amount present was usually negligible. With ammonium salts and minerals (plot 9) the rise was from 0.6 to 13.8 per cent.; again the persistent influence of ammonium salts was manifest, as on plot 5¹ the rise was from 1.0 to 4.6 per cent. and on plot 5² from 5.6 to 20.2 per cent. With nitrogen as nitrate of soda the annual fluctuation is not proportionally very great, but again a big increase occurred in 1922. With dung, with and without minerals, a rhythmic variation occurred, with a tendency to high percentages in 1922.

The use of lime with sulphate of ammonia and minerals has induced much larger and more irregular annual variations, but the increase in 1922 did not occur with this combination of fertilisers.

The climatic conditions in 1922 were not in themselves abnormal, exhibiting a fairly high rainfall, plenty of sunshine and rather low mean temperature. In 1921, however, severe drought persisted throughout the year, affecting the aftermath so seriously that no second crop could be cut. On certain plots, notably those receiving past or present treatment with ammonium sulphate without lime, so much damage was done that the succeeding 1922 crop was abnormally low, and it was on these plots that the proportion of *Alopecurus* rose most. This affords further evidence of the comparative indifference of *Alopecurus* to diverse types of season, as the adverse conditions of drought reduced it so much less than other

species that its proportion in the herbage was spectacularly increased. On the limed part of plot 9, and on other plots where the yield was not seriously depressed, the proportion of *Alopecurus* remained unaffected.

This indifference of *Alopecurus* to season is of special interest in view of its sensitiveness in other respects, as poverty and acidity reduce it to a low ebb, whereas heavy feeding and lime encourage it greatly.

Anthoxanthum odoratum (Tables II and VII). Sufficient quantities to show significant response to season only occur in the presence of sulphate of ammonia and minerals without lime. The yearly variation was considerable between 1919 and 1924, after which the proportion became fairly stabilised till 1929. In that year extreme winter frost was followed by three months of exceptionally low rainfall from January to March, and the herbage on the unlimed sulphate of ammonia plots was practically killed. As recovery took place in succeeding years *Holcus lanatus* dominated the situation, *Anthoxanthum* being practically suppressed. The species was exceptionally abundant in 1923, rising from 8.4 to 42.6 per cent. and from 2.6 to 19.7 per cent. on plots 9 and 18 respectively, falling again heavily in 1924. The most characteristic feature of 1923 was its scarcity of bright sunshine associated with a low mean temperature during the later half of the growing season. The total bright sunshine from January to June was only 587 hours, compared with an average of 784 hours during the ten years 1918–28.

Arrhenatherum avenaceum (Tables II and VII). Good manuring, particularly with lime, is essential to the well-being of this species. With nitrogenous and mineral manures (plots 9 and 14) the annual fluctuations in percentage are often relatively large, but it is difficult to link up the variations with special types of season. Without lime the proportion with ammonium sulphate is usually low, but in 1919 it rose to the high figure of 47 per cent., in a year characterised during the first three months by abnormally high rainfall, low sunshine and low mean temperature. With lime, and with nitrate of soda, this increase did not occur, indicating some correlation between the influence of soil acidity and the particular seasonal conditions. With minerals, season has little effect and the increase to 28 per cent. in 1925 is noteworthy in that it only occurred in the presence of lime, whereas without lime *Arrhenatherum* was unable to take advantage of the opportunity for increase offered by the sudden drop in the proportion of *Lathyrus*.

With dung the fluctuations show no definite correlation with season, but with minerals also (plot 20) rhythmic changes occur comparable with those of the grasses as a whole.

194 *Influence of Season on Botanical Composition of Grassland*

Avena pubescens (Tables II and VIII). Although rarely present in any great quantity, *Avena* is outstanding in its persistent regularity in proportion from year to year, particularly where no lime has been applied. With lime slightly more variation occurs, but there is a definite tendency towards stabilisation of proportion.

Bromus mollis has always been recognised as being most variable with season. Frequently it is quite insignificant on all the plots on which it occurs, while in other years it may suddenly increase, far more on certain plots than on others. In 1903 *Bromus* constituted 23 per cent. of the herbage with heavy nitrate and minerals, whereas in 1919 it was only 0.5 per cent. During the period of annual analyses *Bromus* at first was very insignificant, being less than 1 per cent. on any plot, but the years 1921, 1922 and 1923 were favourable to its growth on plots 7, 14 and 20 (Table III).

Table III.

Percentages of Bromus mollis in Park Grass herbage.

	Plot 7		Plot 14		Plot 20		
	U.	L.	U.	L.	U.	L.L.	H.L.
1920	No analysis		0.5	0.2	0.3	0.1	0.1
1921	0.1	2.6	1.0	1.5	0.5	1.2	0.7
1922	0.2	5.4	4.6	6.5	1.7	2.2	2.8
1923	0.1	7.9	3.7	15.2	2.4	2.4	2.9
1924	—	—	—	—	—	0.03	—

U. = unlimed; L. = limed; L.L. = light limed; H.L. = heavy limed.

With minerals (plot 7) there was no increase without lime, but a progressive rise with lime, whereas with minerals together with nitrate of soda or dung the improvement was manifest also in the absence of lime. After 1923 the species reverted to insignificance and no marked increase has since been recorded.

Dactylis glomerata (Tables II and VIII). Seasonal variations are usually slight, and if for any reason a big change does occur the tendency is for the proportion to remain at the new level in future years. In 1925 with minerals (plot 7) and with nitrate (plot 17) *Dactylis* responded favourably to the conditions which reduced *Lathyrus*, and (on plot 7) showed only a gradual decrease over the next eight years, with no seasonal drop even with the unfavourable conditions of frost and drought in 1929. The familiar rhythmic cycle was shown on both dunged plots, the highest maxima being reached in 1919 and 1927.

*Festuca ovina*¹ (Tables IV and VIII). The behaviour of *Festuca* was

¹ Throughout this paper *Festuca ovina* is used in a group sense, including also *F. rubra* and *F. duriuscula*, as in Bentham and Hooker's *Handbook of the British Flora*.

comparatively uniform over a wide range of manuring. In nearly all cases a steady increase in percentage occurred from 1919 to 1923, followed by a sudden drop in the next year, when many other results were erratic. The only exception was on the plots on which ammonium salts had been discontinued, where *Festuca* remained at a high level, notably on the area now left unmanured. Apparently *Festuca* is usually less affected by season than by other environmental factors, though occasionally a particular combination of meteorological conditions may induce a direct response which, however, is still closely influenced by manuring and soil conditions.

Table IV.

Percentage of grasses in Park Grass herbage.

Plot ...	3		5 ¹	5 ²	7		9	
	U.	L.	U.	U.	U.	L.	U.	L.
<i>Festuca ovina.</i>								
1919	7	5	46	20	7	5	4	6
1921	13	13	40	28	18	10	10	8
1922	13	10	15	17	24	13	11	5
1923	20	18	n.	n.	28	10	12	13
1924	10	8	38	20	11	2	8	3
1925	11	8	n.	n.	9	3	4	2
1926	8	6	28	14	n.	n.	2	3
<i>Holcus lanatus.</i>								
1919	9	8	0.6	1	4	2	12	0.8
1921	11	9	0.3	2	12	2	30	2
1922	3	2	0.3	1	4	2	32	1
1923	4	3	n.	n.	2	1	12	0.2
1924	2	2	0.2	0.1	1	0.5	14	0.2
1925	8	5	n.	n.	6	0.7	40	0.3
1926	7	6	1	4	n.	n.	51	3

U. = unlimed; L. = limed; n. = no analysis.

Holcus lanatus (Tables IV and VIII). *Holcus* is probably more directly responsive to season than any other species present. This may be partly due to its habit of starting into growth early in the year, when plots containing much *Holcus* are green while all others are still brown and lifeless. With moderate manuring the quantity is usually small, marked increases occurring in 1921, 1925 and 1927, when the seasons were relatively warm. On the acid soil produced by sulphate of ammonia and minerals (plot 9) *Holcus* has of recent years come to dominate the situation. In 1919, 1923 and 1924 it was depressed to below 15 per cent., the common characteristic of these years being low temperatures in either the early or later part of the growing season. After this, the proportion having increased to 75 per cent. during a series of favourable years, the whole herbage was wiped out by the frost and drought of 1929.

196 Influence of Season on Botanical Composition of Grassland

The earliness of *Holcus* then gave it an advantage and it returned 100 per cent. strong to the exclusion of all else. In 1931, again a year of low temperatures and rainfall during the first quarter, a certain amount of *Festuca* and *Agrostis* reasserted themselves and *Holcus* was reduced to 76 per cent., but this did not last and since then the entire herbage has consisted of *Holcus* for three successive years. This increasing dominance of *Holcus* cannot be associated with increasing acidity, as the soil reaction has remained unchanged at 4.0 since 1903. It is rather a question of increasing competition, as *Holcus* does not mind acidity while the other species tend to be adversely affected. If *Holcus* is benefited by a favourable season it is able to maintain its improved position because

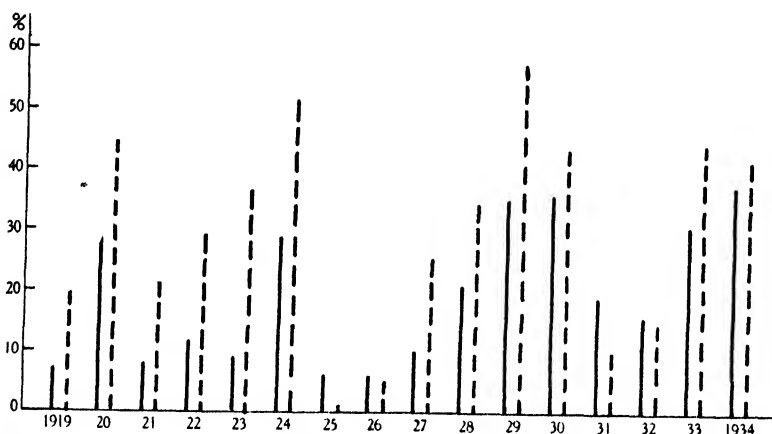


Fig. 4. Percentage of *Lathyrus pratensis* on plot 7 (minerals), 1919-34. — Unlimed. - - - - Limed. (For 1920, and 1926-34 the figures are those for total Leguminosae, of which nearly all was *Lathyrus*.)

the other species are less able to compete effectively and regain their lost ground. It remains to be seen whether further reduction of *Holcus* in unfavourable seasons will ultimately result in a more balanced herbage on the acid plots.

Lathyrus pratensis (Tables V and IX). With continuous mineral manuring (plot 7) *Lathyrus* exhibits more spectacular fluctuations than any other species on the plot. It forms by far the largest proportion of the leguminous plants, especially on the unlimed area, and although complete analyses were only made till 1925 the Leguminosae from the later partial analyses give a true picture of the behaviour of *Lathyrus*. Prior to 1925 the proportion had steadily risen, rather irregularly to 28 per cent. without lime, and fairly steadily to 51 per cent. with lime,

but in this year the figures dropped to 6 and 1 per cent. respectively (Fig. 4). It is difficult to associate this sudden reduction with any particular seasonal peculiarity, as all the months of the growing season were more or less of an average nature. A second year's depression was followed by a rhythmical rise and fall with maxima in 1929 and 1933, the rate of change being greater in the presence of lime. This response to season was paralleled on the plots receiving dung, where the effect was again most marked with lime.

Centaurea nigra (Table V). On most plots this is not plentiful, but where it occurs in any quantity it shows considerable response in certain seasons, the years 1922 and 1924 being very favourable. In 1922 after a preceding year of drought the increase of *Centaurea* was noticeable wherever it occurred, except with dung, but in 1924 the response was less widespread, being confined to plots without manure or with minerals after complete fertilisers (plots 3, 5²).

Plantago lanceolata (Tables V and IX). From the data available it does not appear that *Plantago* shows any striking variation with season, though the fluctuations are greater on the mineral plots (7 and 15) than on those receiving no manure (plot 3) or nitrate of soda (plot 17). Unfortunately plots 15 and 17, on which the species is most abundant, have only been analysed every other year, and it is therefore impossible to obtain an accurate estimate of response in these cases. The periodic variation in proportion is usually greater on the unlimed areas of the plots.

Table V.

*Percentages of leguminous and miscellaneous plants
in Park Grass herbage.*

Plot	...	<i>Lathyrus pratensis</i>			<i>Centaurea nigra</i>					<i>Plantago lanceolata</i>		
		5 ²	7		3	5 ¹			5 ²	7	3	
			U.	L.		U.	U.	U.				
				U.								L.
1919		1	7	20	6	6	4	3	3		19	12
1921		6	8	21	4	5	7	3	3		8	9
1922		2	12	29	7	21	19	7	6		11	8
1923		n.	9	36	2	4	n.	n.	1		11	7
1924		11	28	51	9	9	14	6	0.5		10	5
1925		n.	6	1	3	3	n.	n.	2		15	7
1926		7	n.	n.	3	4	6	3	n.		17	9

U. = unlimed; L. = limed; n. = no analysis.

Rumex acetosa (Table IX). The quantity is usually small and relatively stable except in a few cases where manurial treatment has changed, when an occasional year of abundant *Rumex* has occurred. With complete

198 *Influence of Season on Botanical Composition of Grassland*

fertilisers without phosphate (plot 18) the years 1919 and 1926 were favourable, otherwise response to season is not very marked in most years.

B. RATE OF CHANGE DUE TO LIMING.

The system of liming instituted in 1903 has demonstrated fully the radical change in the constitution of grassland herbage brought about by the addition of lime to other fertilisers. As, however, complete botanical analyses were not made till eleven years afterwards, no quantitative information was gained as to the rate of response of different species. A few plots which had previously been omitted from the scheme have since been dressed with lime from 1920 onwards, and the changes in yield and botanical composition investigated. The effect on yield has been discussed previously (3, 4), and the analyses now under consideration indicate the response of the component species to the change in conditions.

(1) *Response of main groups of grasses, leguminous and miscellaneous plants to liming.*

With complete manures, whether supplied as dung or artificial fertilisers, the balance of the three main groups was not affected by liming, the variations from year to year being quite irregular, but with one-sided manures a definite bias in one direction appeared sooner or later. With minerals after nitrate of soda (plot 15) liming caused decrease in the proportion of miscellaneous plants, which set in immediately, and an increase in the leguminous plants after a single year of depression, the differences due to liming often being very considerable. This is parallel to the effect of lime on plot 7 which has always received minerals. The behaviour of the grasses for several years was more erratic, but since 1927 the proportion on the limed area has definitely been the lower. It is possible that the grasses are less influenced than the other two groups, and that the balance between the latter determines the relative proportion of grasses on the limed and unlimed areas.

With nitrate of soda alone (plot 17) the interplay was almost entirely between the grasses and miscellaneous plants, as the Leguminosae were merely raised by liming from a negligible amount to a maximum of 2 per cent. Here the issue was clear-cut from the first, grasses being improved and the miscellaneous species discouraged consistently every year.

It appears, therefore, that alterations caused by liming in the proportions of the main groups usually manifest themselves at once, though the effect may become accentuated with time. It is also possible for one

or more groups to be consistently affected, and for either or both the others to show no direct response for several years, if at all.

(2) *Response of individual species to liming.*

Although liming may not have any definite influence on the proportion of the three main groups with certain combinations of fertilisers, the relative percentage of the individual species within those groups may be considerably affected.

The analyses show that while some species respond to liming as soon as the first application is made, others may remain comparatively unaffected for several years or even till the second dressing is given. Very occasionally the primary direction of response may be reversed after the later applications. For purposes of reference in considering these changes the available pH value of the soils may be of interest (5).

Table VI.

pH value of Park Grass soils. Lime applied 1920, 1924, 1928, 1932.

	Plot	U.	L.		Plot	U.	L.L.	H.L.
1923	14	6.38	6.74	1923	18	4.46	4.69	5.22
	15	5.54	6.13		19	5.75	5.71	6.22
	17	6.31	6.81		20	6.00	6.15	6.58
1931	17	5.9	6.5					
1933	17	6.0	7.2					

U. = unlimed; L. = limed; L.L. = light limed; H.L. = heavy limed.

Agrostis vulgaris (Table VII). This was consistently reduced by adequate liming, though in the first years the reduction was often small, sometimes increasing after later applications (plots 15 and 18). With dung (plot 19) the lighter dressing of lime was insufficient to cause any reduction even after the third application, but where artificials were also present decrease occurred after the second dressing.

Alopecurus pratensis (Table VII). The response to liming was closely associated with the manuring, showing increase with sulphate of ammonia and minerals and with minerals alone, decrease with nitrate of soda and minerals, while no consistent behaviour was evidenced in the presence of dung. With nitrate of soda alone no effect was shown for at least eight years, when a possible slight depression set in. The general tendency was for the response in either direction not to be evident for at least two years after the first application of lime, except with minerals alone when no delay occurred. In several seasons the lighter dressings of lime induced the largest proportions of *Alopecurus* in the presence of sulphate of ammonia and minerals.

200 Influence of Season on Botanical Composition of Grassland

Table VII.

Percentage of grasses in Park Grass herbage.

Plot...	14		15		17		18			19			20		
	U.	L.	U.	L.	U.	L.	U.	L.L.	H.L.	U.	L.L.	H.L.	U.	L.L.	H.L.
<i>Agrostis vulgaris et alba.</i>															
1919	0.5	n.	11	n.	6	n.	18	n.	n.	7	n.	n.	7	n.	n.
1920	—	0.2	n.	n.	n.	n.	43	45	35	18	15	15	11	15	6
1921	—	1	15	9	6	5	51	41	42	22	18	14	13	10	5
1922	0.2	0.8	n.	n.	n.	n.	44	36	31	13	16	10	10	11	5
1923	—	0.6	13	14	9	4	48	28	27	14	15	10	10	13	5
1924	0.3	0.3	n.	n.	n.	n.	72	37	26	13	18	8	15	9	4
1925	0.3	—	17	8	3	2	63	37	14	11	10	3	14	4	3
1926	n.	n.	n.	n.	n.	n.	47	18	12	9	9	3	10	5	4
1927	n.	n.	12	6	6	4	75	22	10	5	5	2	4	3	2
1928	n.	n.	n.	n.	n.	n.	59	18	5	6	5	2	4	4	1
<i>Alopecurus pratensis.</i>															
1919	54	n.	30	n.	13	n.	5	n.	n.	22	n.	n.	30	n.	n.
1920	48	53	n.	n.	n.	n.	6	4	6	16	15	22	27	22	30
1921	34	33	8	20	12	10	3	5	5	13	20	16	19	22	31
1922	58	42	n.	n.	n.	n.	10	25	17	22	20	19	23	24	33
1923	42	24	8	15	13	10	5	10	8	16	16	15	29	17	25
1924	44	29	n.	n.	n.	n.	5	23	22	17	16	17	16	27	21
1925	36	19	10	28	14	13	4	14	14	16	22	24	17	15	19
1926	n.	n.	n.	n.	n.	n.	6	25	25	29	30	27	27	22	26
1927	n.	n.	9	14	14	14	4	22	19	26	28	17	30	19	23
1928	n.	n.	n.	n.	n.	n.	5	46	23	33	43	21	46	31	24
<i>Anthoxanthum odoratum.</i>															
1919	0.4	2	3	n.	7	n.	3	n.	n.	4	n.	n.	1	n.	n.
1920	—	—	n.	n.	n.	n.	6	7	5	5	4	2	0.8	2	0.7
1921	—	0.6	5	2	9	3	8	4	4	10	8	3	1	6	2
1922	—	—	n.	n.	n.	n.	3	0.7	1	4	2	0.6	0.4	1	0.3
1923	—	0.1	4	2	7	3	20	2	3	12	9	1	0.3	6	1
1924	—	—	n.	n.	n.	n.	4	0.1	—	4	7	0.6	1	1	0.2
1925	—	—	3	1	7	0.9	3	0.1	—	10	5	0.8	1	2	0.4
1926	n.	n.	n.	n.	n.	n.	4	0.1	0.1	8	8	0.7	1	4	1
1927	n.	n.	4	0.9	11	3	3	0.1	—	9	9	0.5	0.6	3	0.8
1928	n.	n.	n.	n.	n.	n.	7	0.5	—	12	8	0.7	2	6	0.7
<i>Arrhenatherum avenaceum.</i>															
1919	23	n.	1	n.	0.3	n.	2	n.	n.	8	n.	n.	5	n.	n.
1920	37	30	n.	n.	n.	n.	0.5	0.8	0.5	1	4	5	6	2	2
1921	48	41	3	4	—	—	0.7	2	2	8	0.3	14	10	10	4
1922	25	25	n.	n.	n.	n.	1	2	3	11	2	5	7	6	3
1923	33	35	1	3	—	0.4	0.1	2	3	9	2	7	6	12	2
1924	39	40	n.	n.	n.	n.	0.3	2	10	11	1	8	6	10	0.4
1925	40	54	1	1	0.5	—	1	7	1	19	3	15	9	33	4
1926	n.	—	n.	n.	n.	n.	2	9	11	12	6	22	18	24	8
1927	—	—	9	5	1	2	2	10	19	18	6	22	25	31	7
1928	n.	n.	n.	n.	n.	n.	0.4	3	18	8	3	16	11	9	4

U. = unlimed; L. = limed; L.L. = light limed; H.L. = heavy limed;
n. = no analysis; — = missing or below 0.1 %.

The behaviour of *Alopecurus* is closely associated with soil reaction. With low pH, as on plot 18, pH 4.6, and plot 15, pH 5.54, liming is beneficial to the proportion of the species. As neutrality is approached the benefit disappears (plot 17, pH 6.31; plot 19, pH 5.75; plot 20, pH 6.00), or an adverse response occurs (plot 14, pH 6.38) giving results which exactly parallel those obtained by earlier treatment of the other plots on the area.

Anthoxanthum odoratum (Table VII). This is very impatient of adequate dressings of lime and in all cases showed an immediate reduction, rapidly being decreased to an almost negligible quantity. Lighter dressings were somewhat slower in action with sulphate of ammonia and minerals, though they eventually had the same effect, but on the dunged plots the smaller quantity of lime was insufficient to bring about any reduction.

Arrhenatherum avenaceum (Table VII). This is so remarkably inconsistent in its behaviour that it is probable that the action of lime in any season is often profoundly influenced by other factors. With nitrate of soda and minerals (plot 14) a depression at the beginning due to liming was gradually converted into a beneficial effect in later years, but as no analyses have been made since 1925 it is unknown whether this has persisted. At the present time, however, *Arrhenatherum* forms the major part of the herbage on both the limed and unlimed areas of this plot, so that it is unlikely that any very striking effect of liming is manifest with this species.

With sulphate of ammonia and minerals the quantity was originally very small, but a gradual increase was induced by liming, until after the second application the proportion rose to 5 per cent. with the light dressing and to 19 per cent. with the heavy dressing. With dung the result is almost inexplicable. With heavy dressings (on plot 19) the response was irregular, either increase or decrease occurring with season, but from the beginning the lighter dressings have been adverse, reducing the proportion of *Arrhenatherum* much below that on the unlimed or heavy-limed sections. With the addition of artificials to dung (plot 20) the heavier lime dressings have been consistently adverse, whereas the lighter dressings have frequently given the best result of all. This was specially so in 1925, when the percentage rose to 33 with light lime compared with 9 and 4 per cent. on the other sections.

Avena pubescens (Table VIII). Liming proved very beneficial as a general rule, the increase usually beginning at once, and becoming more marked after a second application. With nitrate of soda and minerals lime had no effect, and on the dunged plots, also, the lighter dressings were inadequate and induced no response.

Dactylis glomerata (Table VIII). With nitrate of soda lime definitely prejudices the growth of *Dactylis*, the greatest reduction being with nitrate alone. With minerals and ammonium salts, on the contrary, it is much encouraged by lime, though the benefit was not manifested until after the second application (Fig. 5). With dung no response has occurred,

202 Influence of Season on Botanical Composition of Grassland

except for an occasional tendency to an increased proportion with the lighter dressing.

Festuca ovina (Table VIII). Liming had no consistent effect on *Festuca* except with nitrate of soda, when it caused an immediate and marked increase. A similar result had previously been obtained with the same amount of nitrate together with minerals. With the double dressing of nitrate and minerals (plot 14) the negligible amount of *Festuca* remained uninfluenced, possibly because of undue competition from the larger species of grass encouraged by this manuring.

Holcus lanatus (Table VIII). Liming caused a general tendency to reduction throughout, except on plot 20 when it was quite ineffective. On plot 18 (ammonium salts and minerals) the effect was not a true

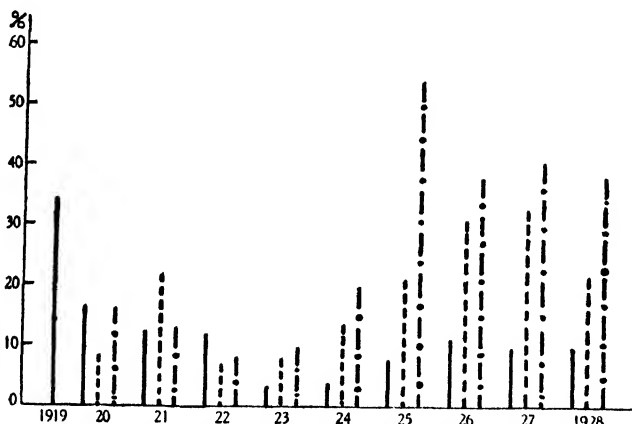


Fig. 5. Percentage of *Dactylis glomerata* on plot 18 (ammonium salts and minerals without phosphate), 1919-28. — Unlimed. - - - - Light limed. . . . Heavy limed.

reduction but was due to the failure of the species, in the presence of lime, to respond to favourable environmental conditions after the abnormal season of 1924, whereas on the unlimed area the proportion increased considerably.

Poa pratensis. This was primarily insignificant or absent on all plots, but with ammonium salts and minerals (plot 18) it showed an immediate and progressive increase with lime, responding equally to both light and heavy dressings, reaching a maximum of 7 per cent. in some years.

Lathyrus pratensis (Table IX). No consistent response to liming was shown except with mineral manures where it was benefited immediately. The difference in the proportion of *Lathyrus* with and without lime fluctuates widely from year to year, as in 1927 the variation was from 5 per cent. unlimed to 26 per cent. limed, whereas in 1923 there was a

Table VIII.
Percentages of grasses in Park Grass herbage.

Plot ...	14		15		17		18			19			20		
	U.	L.	U.	L.	U.	L.	U.	L.L.	H.L.	U.	L.L.	H.L.	U.	L.L.	H.L.
<i>Avena pubescens.</i>															
1919	4	n.	2	n.	5	n.	0.1	n.	n.	3	n.	n.	10	n.	n.
1920	0.2	1	n.	n.	n.	n.	—	—	—	3	4	5	11	10	14
1921	2	4	3	3	4	7	—	—	—	4	2	5	12	8	19
1922	0.7	2	n.	n.	n.	n.	0.3	0.4	1	2	1	3	8	5	7
1923	0.8	5	2	3	3	10	—	0.1	—	2	3	4	8	5	12
1924	0.4	3	n.	n.	n.	n.	—	—	—	1	2	6	5	5	10
1925	0.3	0.1	2	6	2	16	—	—	—	2	3	7	6	3	16
1926	n.	n.	n.	n.	n.	n.	—	0.3	1	2	3	7	6	7	16
1927	n.	n.	3	11	3	17	—	0.1	0.2	3	4	11	5	8	24
1928	n.	n.	n.	n.	n.	n.	—	—	—	3	3	12	7	9	29
<i>Dactylis glomerata.</i>															
1919	3	n.	5	n.	8	n.	34	n.	n.	16	n.	n.	12	n.	n.
1920	5	3	n.	n.	n.	n.	17	8	16	11	9	9	10	8	9
1921	5	3	5	2	5	11	12	22	13	6	16	9	6	9	9
1922	3	2	n.	n.	n.	n.	12	7	8	5	15	12	9	11	8
1923	5	0.6	3	2	7	4	3	8	10	5	6	4	6	4	3
1924	3	1	n.	n.	n.	n.	4	13	19	7	4	5	6	5	5
1925	7	3	10	6	28	15	8	21	53	9	21	18	10	13	9
1926	n.	n.	n.	n.	n.	n.	11	31	37	14	15	16	11	14	13
1927	n.	n.	16	5	24	7	10	32	40	17	26	17	15	18	14
1928	n.	n.	n.	n.	n.	n.	9	21	38	10	11	9	7	7	6
<i>Festuca ovina.</i>															
1919	5	n.	7	n.	4	n.	4	n.	n.	6	n.	n.	4	n.	n.
1920	—	2	n.	n.	n.	n.	14	5	12	12	12	15	10	10	10
1921	0.1	5	22	20	12	21	12	6	7	12	10	15	9	9	7
1922	0.2	5	n.	n.	n.	n.	9	10	12	18	15	15	16	14	13
1923	0.2	9	21	23	17	35	18	26	31	13	15	17	14	15	21
1924	0.1	7	n.	n.	n.	n.	5	6	4	8	10	13	8	6	10
1925	—	0.1	9	10	6	22	6	5	5	8	5	7	4	1	4
1926	n.	n.	n.	n.	n.	n.	3	2	2	4	3	4	3	3	5
1927	n.	n.	8	8	5	21	3	3	3	6	3	11	3	3	5
1928	n.	n.	n.	n.	n.	n.	6	3	3	8	4	14	5	6	9
<i>Holcus lanatus.</i>															
1919	—	n.	6	n.	11	n.	2	n.	n.	2	n.	n.	7	n.	n.
1920	—	—	n.	n.	n.	n.	1	8	2	2	2	2	3	5	7
1921	—	—	11	5	16	12	4	3	5	5	7	2	10	10	8
1922	—	—	n.	n.	n.	n.	0.9	2	1	1	2	0.5	3	4	3
1923	—	—	2	2	3	3	0.2	1	2	3	2	0.2	2	2	2
1924	—	—	n.	n.	n.	n.	0.2	0.2	—	1	2	0.8	2	2	1
1925	—	—	10	4	10	6	0.8	0.4	0.3	6	2	2	4	4	5
1926	n.	n.	n.	n.	n.	n.	3	1	2	4	2	1	7	7	8
1927	n.	n.	12	3	12	10	2	3	2	3	2	1	7	7	7
1928	n.	n.	n.	n.	n.	n.	8	0.8	2	5	3	0.9	4	8	5

U. = unlimed; L. = limed; L.L. = light limed; H.L. = heavy limed;
 n. = no analysis; — = missing or below 0.1 %.

slight but transitory depression on the limed areas. With dung and artificials the lighter dressings encouraged *Lathyrus* more than the other two treatments, but this was merely temporary and disappeared as soon as the second dressing of lime was applied.

Trifolium repens. This is usually present only in small amount in the first cut of hay, as its tendency is to develop rather late. In 1923, 1929 and 1933 unusual quantities occurred on the limed part of plot 15, with

204 Influence of Season on Botanical Composition of Grassland

minerals, reaching the high figures of 19 per cent. in 1929, a year of abnormal seasonal conditions. This year, also, large amounts were recorded from the dunged plots with lime, though this increase did not appear on any other occasion. Owing to the late habit of the species it is difficult to ascertain the true response of *Trifolium repens* to lime from the data available.

Achillea millefolium (Table IX). The amount was only significant with minerals, the effect of lime being to keep the proportion constant, preventing it from responding to any seasonal conditions that were advantageous to the species, as occurred in 1929.

Table IX.

Percentages of leguminous and miscellaneous species
in Park Grass herbage.

Plot...	<i>Lathyrus pratensis</i>									<i>Rumex acetosa</i>		
	15		19			20				18		
	U.	L.	U.	L.L.	H.L.	U.	L.L.	H.L.		U.	L.L.	H.L.
1919	5	n.	6	n.	n.	5	n.	n.		24	n.	n.
1920	n.	n.	12	18	8	4	15	5		9	15	18
1921	8	18	5	2	3	3	5	4		6	11	14
1922	n.	n.	7	6	7	1	8	4		4	6	8
1923	15	11	7	9	15	2	10	5		2	13	12
1924	n.	n.	19	18	21	22	17	30		2	10	10
1925	4	14	4	3	1	10	2	4		10	11	5
1926	n.	n.	2	1	0.9	4	1	3		21	9	5
1927	5	26	1	1	1	2	0.5	3		1	1	0.8
1928	n.	n.	2	1	1	3	2	5		3	2	1
1929	16	25	-	-	-	-	-	-		-	-	-
1931	5	-	-	-	-	-	-	-		-	-	-
1933	8	14	-	-	-	-	-	-		-	-	-

Plot ...	<i>Achillea millefolium</i>		<i>Plantago lanceolata</i>			
	15		15		17	
	U.	L.	U.	L.	U.	L.
1919	5	n.	4	n.	24	n.
1921	3	2	7	4	29	18
1923	2	1	13	5	27	16
1925	7	2	15	8	17	11
1927	6	3	6	5	16	8
1929	16	4	6	4	23	15
1931	8	-	2	-	-	-
1933	1	0.9	4	10	16	17

U. = unlimed; L. = limed; L.L. = light limed; H.L. = heavy limed;
n. = no analysis; - = missing or below 0.1 %.

Plantago lanceolata (Table IX). With minerals and with nitrate of soda liming caused a marked decrease in the proportion of *Plantago*, but where the amount was originally insignificant the species remained unaffected and was not completely eradicated by lime.

Rumex acetosa (Table IX). Relatively small quantities were present on most plots and showed a general tendency to reduction with lime except with ammonium sulphate and minerals without phosphate (plot 18). In the latter case lime apparently induced a marked increase in *Rumex* for the first five years, after which a reaction set in and the effect of liming became variable with season, tending to decrease the species. Care is necessary in interpreting these results owing to the large percentage of *Rumex* that was present in 1919. In that year the sample was drawn from the whole plot, and, in 1920 from the three sections as divided for liming. It is quite possible that the *Rumex* was originally distributed unevenly over the plot, being more prevalent on the areas which later received lime, and that the figures in the first few years are more or less a reflection of the original proportion of *Rumex* of the plot. The change towards a decrease with lime which set in after the second application may therefore present the real state of affairs, i.e. that with minerals and ammonium salts lime tends to decrease the proportion of *Rumex*. If this be true, then the reaction corresponds with the earlier results on the other plots with similar manuring.

Shade is another factor which influences the balance of species in herbage, as is shown by a section of the limed area of plot 14 (nitrate and minerals) which is in the shadow of a large oak tree during the earlier hours of the day. The proportion of the main groups is not usually much affected, as it is determined in this case by the dominating influence of the manurial treatment, though a certain increase in Leguminosae is sometimes registered. The individual species, on the contrary, show striking variations (Table X). *Festuca ovina* and *Avena pubescens* are increased by shading from a negligible quantity to about 15 per cent. in a typical year, while *Arrhenatherum* shows a correspondingly heavy drop, *Poa trivialis* also being reduced. *Alopecurus* and *Dactylis*, although present in quantity, seem to be indifferent to the effect of shading.

Table X.

Effect of shade on the botanical composition of herbage, 1925.

(Plot 14, nitrate of soda and minerals, with lime.)

	Unshaded %	Shaded %
Increased: <i>Avena pubescens</i>	0.1	16.0
<i>Festuca ovina</i>	0.1	16.9
<i>Lathyrus pratensis</i>	Trace	5.7
Indifferent: <i>Alopecurus pratensis</i>	18.9	22.0
<i>Dactylis glomerata</i>	3.0	2.7
Decreased: <i>Arrhenatherum avenaceum</i>	54.0	29.6
<i>Poa trivialis</i>	6.6	Trace

206 *Influence of Season on Botanical Composition of Grassland*

The occasional increase in leguminous plants, such as occurred in 1920 and 1925, is entirely due to *Lathyrus pratensis*, but the proportion has never exceeded 7 per cent.

The available numerical data only refer to the one plot, but observations in the field corroborate the fact that shading exercises a marked influence on the botanical composition of herbage, though the direction of response of any individual species may vary according to soil conditions and manurial treatment.

SUMMARY.

The botanical composition of the herbage of grassland under constant manurial treatment varies considerably from year to year.

With complete fertilisers including nitrogen and minerals the relative proportions of the three main groups of species, *i.e.* grasses, leguminous and miscellaneous plants, are not usually much affected by season, though the individual species do vary, but with one-sided fertilisers and on unmanured areas wide fluctuations occur in the percentage of these groups. No correlation can be traced between the annual variations in the yield and the botanical composition of the herbage, except for some suggestion of association between high yield and high percentage of leguminous plants with long-continued mineral manuring.

The variations of individual species occur on all plots. They may be caused by direct or indirect response to season and are much influenced by the type of manuring. It is often difficult to determine whether a marked increase or decrease of a species in any year is due to climatic conditions being beneficial or detrimental to that particular species. It may be that the real effect is on other constituents of the herbage which change so much that the proportion of the species under consideration is radically affected (*cf.* *Alopecurus* in 1922). In some cases, especially with organic fertilisers, the main groups and also certain species (as *Alopecurus*, *Arrhenatherum*, *Dactylis*) show a tendency to rhythmic changes with season, rising and falling over a period of years. In other cases the fluctuations are more abrupt and irregular, sometimes being exaggerated in the presence of lime.

The application of lime to plots with long-established manurial treatment does not affect the balance of the three main groups with complete fertilisers, but with one-sided manures a definite bias in one direction appears sooner or later. Individual species usually respond to lime at once, showing a change of proportion at the first succeeding cut, but under certain soil conditions a delay may occur until a second dressing

has been given. It would appear that the maximum effect of liming is reached within a few years from the first application, after which fluctuations with season may again become more obvious.

Shade is also a factor which influences the balance of species in herbage. The available data is limited to a single plot, but indicates that certain species may be greatly increased or decreased as a result of shading, whereas the proportion of other species may not be affected.

REFERENCES.

- (1) LAWES, J. B., GILBERT, J. H. and MASTERS, M. T. (1882). Results of experiments on the mixed herbage of permanent meadow. Part II. The botanical results. *Philos. Trans.* Part IV, pp. 1181-1413.
- (2) BRENCHELEY, W. E. (1924). *Manuring of Grassland for Hay*. Pp. 144. Longmans, Green and Co.: Rothamsted Monographs.
- (3) — (1925). The effect of light and heavy dressings of lime on grassland. *J. Minist. Agric.* XXXII, 504-12.
- (4) — (1930). The varying effect of lime on grassland with different schemes of manuring. *Ibid.* XXXVII, 663-73.
- (5) CROWTHER, E. M. (1925). Studies on soil reaction. IV. The soil reaction of continuously manured plots at Rothamsted and Woburn. *J. agric. Sci.* xv, 222-36.

(Received January 28th, 1935.)

MIXED CROPPING IN PRIMITIVE AGRICULTURE

HUGH NICOL

(Department of Bacteriology, Rothamsted Experimental Station)

In previous publications [1, 2, 3] the author has stressed the importance to non-leguminous plants of leguminous plants growing in mixture with them. There is a considerable amount of evidence, much of which has been reviewed in [2], that in mixed cropping the leguminous component of a mixture can act as provider of nitrogen for the non-legume. The author [2] has already mentioned the practice common near Cawnpore of growing gram (*Cicer arietinum*) **P**¹ and wheat together. Dr. H. H. Mann recently directed² the author's attention to the fact that the culture of mixed crops is much more widespread than would be inferred from that isolated example.

Of *bajri* (*Pennisetum typhoideum*) in the Bombay Deccan, Mann [5] wrote:

Its real importance would be better understood if the area under the so-called *bajri* mixture were taken. The *bajri* mixture contains several leguminous crops, and hence can be grown year after year without affecting the fertility of the land to any great extent. As a result there is little of ordinary rotation practised. . . . It [*bajri*] is never sown alone, but always mixed with one or more pulses and several other seeds. . . . The special feature of the *bajri* mixture in this village is the very large number of [varieties of] seeds which are mixed with the *bajri* before sowing. . . . The seeds sown are stated to vary according to the soil. . . . The mixture is sown with a three-coulter drill, and at the same time a fourth row is sown either with *tur* [*Cajanus indicus* **P**] or *kulthi* [*Dolichos biflorus* **P**] according to the nature of the soil.

Mann added a table showing the proportions of seed of ten species in eight mixtures analysed after sampling at time of sowing. No sample contained fewer than four species in addition to that sown in the fourth row.

Mann gave sociological and agricultural reasons to explain this custom of growing mixtures rather than single crops.

None of the pulses, the oil-seeds, or the other constituents of the mixture are ever sown as independent crops. . . . In practice, it is a fact that whenever the crop of *bajri* is good, the crop of pulses is poor [and so on]. The land is mostly very poor, and it is hence not possible to follow a system of rotation. Hence, the mixture, which answers, in part at any rate, the same purpose, is resorted to.

It is noteworthy that the poorer the land, the smaller the proportion of *bajri*; one infers, without being certain, that the richer soils require a smaller admixture of legumes.

The question of the roles of animal manures and of leguminous plants

¹ Throughout the paper the less common leguminous plants are distinguished by **P** after their names. In quotations, **P** is necessarily an interpolation; in other cases the author's own interpolations are distinguished by square brackets.

² By supplying books [4], [5], and [6], and the quotation [7]. Many other references could be given.

in rotations is of considerable interest. There is evidence that one may supplement or replace the other, but as yet no experiment has been designed to test these points specifically. Since in native Indian agricultural economy, cattle and other organic manures are usually insufficient in amount, and artificials can rarely be bought, the value of the leguminous component of mixed crops emerges even more clearly than it does from a consideration of our home pastures. Voelcker [8] is definite:

It is quite a mistake to suppose that Rotation is not understood or appreciated in India. The contrary is the case. Frequently more than one crop at a time may be seen occupying the same ground, but one is very apt to forget that this is really an instance of rotation being followed. . . . The next year the same 'mixed crops' may be grown again, and thus to the casual observer it might appear that continuous cropping was being practised. This, however, is not so, for there is a perfect rotation of cereal and legume. (Para. 304, 'Mixed crops'.)

In his survey of agricultural India in 1887, Wallace [9] devoted a special chapter to 'Rotations and mixed crops'. He wrote:

The growth of mixed crops is a wide-spread practice which is well worth consideration and study. . . . The advantages under Indian conditions are distinctly great. . . . There is but one explanation of the existence of these practices [of mixed cropping], viz., that they have been found advantageous after long experience and much careful consideration on the part of a body of workers who, for power of observation and an intelligent interest in and knowledge of everyday occurrences, would put to shame those classes which hold a corresponding position in educated Europe.

Wallace set forth, as did Mann [4, 5], the reasons usually given for the benefits of mixed cropping, and stated that the roots of different species possess different root-habits and different functional powers. The case for studying root-habit in India has been forcibly put by Howard [10] in a chapter especially devoted to 'The Economic Significance of Root-Development'. So far as the author is aware, the only study of root-interactions of crops grown singly and mixed is that made in Austria by Kaserer [11], whose valuable paper has been much neglected. Kaserer noted that there was little or no interpenetration of roots of plants of the same species grown together, however densely, whilst an increasing degree of interpenetration was noted with increasing dissimilarity of two species grown together. A legume and a non-legume showed the maximum of interpenetration; in Kaserer's words: 'Eine Graminee mit einer Leguminose zeigte stets Verfilzung.'

Kaserer's observations have an evident relation to the uptake by one plant, of nutrients produced or made available by another. The author, with Thornton at Rothamsted, has noted the fact that the roots of lucerne and of grass, grown together in sand-pots, are separable with difficulty; whereas no difficulty has been experienced in parting the root-systems of plants of lucerne, grass, peas, and clover, grown in single culture under similar conditions.

To Wallace might perhaps be given the credit of priority already accorded by the author [2] to Leather [12], who wondered from a consideration of the Cawnpore gram **P** and wheat mixture, whether 'the

Papilionaceae are able to assist in any way the plant of another natural order *which is growing alongside them*¹. Wallace wrote [9]:

I am inclined, also, to think that there may be decaying roots or matters thrown off by plants of distinct species, which matters, in the hot climate, become available within the period of growth of a given mixed crop; and, in the case of a grain crop grown along with a mixture of pulse, we may have more or less of a beneficial action, such as that of the well-known influence of clover root upon a succeeding wheat crop.

Wallace did not recall in this connexion the British pastures, with their leguminous herbage, or the practice of sowing clover in barley. Similar omissions were made by Lawes and Gilbert, Munro and Beaven, and others (Nicol [2]).

The value of having leguminous and other plants and trees in mixed culture with tea has been extensively discussed. Mann [13] has given thorough consideration to the effects, upon growth of tea, of *sau* (*Albizzia stipulata*) P, and several other species of trees, bushes, and crops. He wrote in 1907 [13]: 'It has been suggested that possibly the tea root gets actually in contact with the root of the *Albizzia* tree and draws nourishment from it.' In view of Kaserer's [11] and the author's observations upon *Verfilzung*, it may be hoped that more concentration will be given to the subject of the so-called 'shade' cropping in tea gardens; a study of root-habits should be particularly helpful.

The part played by leguminous 'weeds' under natural, wild, and semi-natural conditions, in maintaining and restoring the level of fertility of soils, is also striking, though it has received relatively little attention. By way of illustration of the perseverance of Nature in building up poor land, it may be remarked that upon the continuously cropped Broadbalk wheat-field at Rothamsted (in theory a pure culture) an abundant growth of wild black medick (*Medicago lupulina*) P frequently occurs on all of the cropped plots receiving no nitrogen or the lowest dressing of nitrogen.¹ The darkening of the otherwise bare stubbles by the green *Medicago* after the 1934 harvest was sufficiently evident to be photographable by the author. Wallace [9] commented upon the richness in legumes of the natural Indian flora, and assigned to them an important part in maintaining fertility in uncropped land.

Apparently it is not essential in Indian agriculture that a mixed crop should include a legume, since the association of *jowar* and safflower (*Carthamus tinctorius*) is recorded by Mann [5], and other examples could be given. Nevertheless, leguminous plants occur in Indian mixtures with great frequency. Mollison [6] brings out this point clearly:

Various pulses, oil-seeds and fibre crops are generally grown with *kharif jowar* [*Sorghum vulgare*, a grain; *kharif* is the name of a season]. In Gujarát there is a greater variety than elsewhere. There, subordinate to *jowar*, we find *tuver* [*Cajanus*

¹ The absence of legumes from plots receiving the higher doses of nitrogen does not imply that the nitrogenous manure was toxic to leguminous plants; it almost certainly meant that ample manuring encouraged growth of the wheat sufficiently to 'smother' the low-growing black medick. Analogous observations on clover undersown in barley were made at Woburn by Dr. H. H. Mann, who agreed with the author that the Broadbalk phenomena helped to furnish an explanation.

indicus P], *guvdr* [*Cyamopsis psoraloides* P], *math* [*Phaseolus aconitifolius* P], *mag* [*P. mungo* P], *chola* [*Vigna catjang* P], *adad* [*Phaseolus mungo* var. *radiatus* P] (all pulses), castors and *tal* [*Sesamum indicum*] (oil-seeds), and *ambádi* [*Hibiscus cannabinus*] and *rozi* cotton (fibre plants). The group of subordinate crops referred to are not often sown all together, but mixed according to the fancy or inclination of the cultivator. In Khándesh, *udid* P [= *adad*] and *ambádi* are ordinarily sown with *jowár*. In the black soil of Surat, *tuver* P is always subordinate to *jowár*, and generally also *mag* P. In the Deccan, on mixed black soil, *tur* P, *ambádi*, *udid* P, and sesamum, and on distinctly light soil, *math* P, *kulthi* P and sometimes niger seed [*Guizotia abyssinica*] are generally subordinate to *jowár*.

Discussing the figures recorded for the acreage of *jowar*, Mollison [6] wrote:

These figures are, to some extent, misleading, because it is the general practice to grow *jowári* and nearly all cereals with a subordinate pulse mixture.

The latter point is borne out in his detailed descriptions, later in the book, of the methods of cultivation of other grains.

The following is an excerpt from an article by Mollison [7], written when he was Inspector-General of Agriculture in India:

The common Indian system of growing mixed crops serves in many respects the purposes of rotation. It is undoubtedly a successful and profitable method, which has done more to uphold the fertility of Indian soils than any other practice. There are very good reasons why it is profitable to grow pulses, oil-seeds, and fibre plants mixed with or subordinate to cereals like *jowar*, *bajri*, or wheat. . . . Pulse crops whether grown alone or in combination with other crops, exercise another beneficial influence in that they enrich the soil with nitrogen, of which element Indian soils require a frequently renewed supply. The common growth of these pulses is a testimony to the fundamental soundness of the traditional agricultural practice of the country. No pulse crop cultivated in India exercises such a general fertilizing effect as *arhar* (*Cajanus indicus*) P. It is grown in every province mixed with other crops: its long tap-root enables it to withstand drought and to search in the subsoil for plant food: it spreads out and grows freely after the cereal to which it is subordinate has been harvested; and nearly all the leaves fall as the plants ripen, thus enriching the surface soil.

Custom varies in different districts. Thus, gram P and wheat or barley, a common mixture in the North-West Provinces, is unknown in the Bombay Presidency [6]. Mann [5], however, states that the cultivation of gram P in Bombay Presidency is intimately associated with that of wheat, gram having generally been considered as the natural rotation crop with wheat.

Regarding native agricultural methods in West Africa, Irvine [14] confirms the praise for mixed cropping already given by observers in India:

Mixed cropping is really a modified form of crop rotation and has several advantages. . . . Sometimes, the two or more crops growing together use different quantities of the available plant food, and their roots go to different depths in the soil. In this respect mixed cropping is more scientific than pure cropping.

An additional advantage claimed by Irvine for mixed cropping is the reduction of damage by insects.

Willis made several references in his book [15] to mixed cultivation and the similarity of its effects to rotation.

Mixture of crops, which seems to bring in its train some of the advantages of rotation, is very common, especially in the more equatorial parts of the tropics, such as southern Ceylon, Malaya, the West Indies, &c. Not only is there . . . mixture of perennial crops, but mixture of annuals is very common in the East: pulses are sown among the grain, different kinds of grain with one another, and so on. Here again the gain is somewhat like that obtained by rotation. . . .

Of 'the wild jungle-like mixture' of trees and vegetables which forms the average native garden throughout southern Asia, Willis wrote:

As pointed out above, it is highly probable that this arrangement gives many of the advantages which have elsewhere to be attained by rotation of crops, and the villager is thus able to grow his familiar foods, &c., on the same ground for an indefinite number of years. Mixture of crops, as well as rotation, requires very careful study in detail before any hasty attempt is made to change immemorial custom.

In the minds of many agriculturists, the customs of the Red Indians of North America are distinguished by their occasional practice of burying a piece of fish in each 'hill' of maize. This is often recalled as an example of primitive manuring. The author was aware of this practice but imagined that the maize was grown in pure culture, not having read or heard anything to the contrary. Consultation of the early part of Carrier's book [16] did not remove this impression, until on page 94 three quotations concerning mixed cropping were found. It is odd that Carrier has not thought it worth while to bring into relief, in his text, the practice of mixed cropping, since many other cultural operations are considered at length by him.

Hariot [17] wrote (*v* having been substituted for *u*):

All the aforesaide commodities for victuall are set or sowed, sometimes in groundes a part and severally by themselves; but for the most part together in one ground mixtly. . . . The ground they never fatten with mucke, dounge or any other thing. . . .

Then their setting or sowing is after this manner. . . . First for their corne. . . . By this meanes there is a yarde spare ground betwene every hole [each containing four seeds of maize]: where according to discretion here and there, they set as many Beanes and Peaze: in divers places also among the seedes of *Macocquer*, *Melden*, and *Planta Solis*.

The ground being thus set according to the rate by us experimented, an English Acre containing fourtie pearches in length, and foure in breadth, doeth there yeeld in crophe or ofcome of corne, beanes, and peaze, at the least two hūdred London bushelles: besides the *Macocquer*, *Melden*, and *Planta Solis*.

Hariot also wrote of the celerity with which the ground was sown; and the abundant mixed crop yielded from ground 'having once borne corne before'. Roanoke, the part of 'Virginia' described by Hariot, is now assigned to North Carolina. Carrier [16] did not quote the first paragraph here cited from Hariot.

From Pinkerton [18], after Carrier [16]:

They (Indians) make heaps like mole hills each about 2½ feet from the others which they sow or plant in April with maize in each heap 5 or 6 grains, in the

middle of May when the maize is the height of a finger or more they plant in each heap 3 or 4 Turkish beanes which they grow up with and against the maize.

Of Indian maize-planting in Virginia in 1606, John Smith wrote [19]:

They make a hole in the earth with a stick, and into it they put foure graines of wheate and two of beanes.

On page 96 of his book *Carrier* has reproduced a picture (ascribed to Le Moyne, 1564), showing Indians planting corn and beans in the same field.

Discussion

The foregoing review is intended to be indicative rather than exhaustive. All the authors consulted agree in assigning a prominent part to mixed cropping in primitive agriculture. No author has been found who has denied its existence. Although several sources consulted (such as the works of Fortune, Huc, King, and others on China and Japan) do not mention mixed cropping unless incidentally or in relation to intensive gardening,¹ it seems probable that in many cases the omission is due to a one-sided orientation. With the Red Indians, for example, the case is clear, yet Carrier did not refer in his own text to mixed cropping as such, and omitted the first paragraph of Harriot given above. Although he claimed that the Red Indians were pioneers of intertillage for keeping down weeds, he did not mention intercropping. On the subject of rotation, Carrier wrote that the Red Indians practised a rotation of fields rather than of crops. His example shows how easy it is to miss a point. Probably the absence of specific remarks upon mixed cropping in the recorded observations of many authors is not to be taken as evidence of absence of the cultivation of mixtures.

Clover and barley, and clover in grassland, have already been referred to, as examples of mixed cropping overlooked in his native land by so astute an observer as Wallace.²

Administrators in India have recognized the fiscal importance of mixed cropping, by drawing up rules for the estimation of the areas to be ascribed to each component of mixtures. It is therefore remarkable that, in spite of repeated recommendations, the agricultural problems underlying the practices of mixed cropping have been so little studied. In

¹ A French observer, Hedde [20], under the heading 'Fruchtwechsel' wrote as follows: 'Die Chinesen verstehen sich dergestalt auf die Unterhaltung der Erde durch die Kombination der veränderten Kulturen, dass man sich zu der Aeußerung veranlasst fühlte: "Der Ackerbau wäre bei ihnen Gartenbau." Sie glauben nicht daran, dass die durch die Erbauung der verschiedenen Pflanzen bewirkte Arbeit die Erde jemals erschöpfe; im Gegentheil nehmen sie an, dass, wenn eine Pflanze einen besondern, ihr nothwendigen Stoff absorbire, sie, als eine Ausgleichung, ein neues Element oder einen natürlichen, einer andern Kultur günstigen Düngstoff zurücklasse.'

Fortune [21] wrote of mixed crops in Chinese tea plantations: 'Another reason for the practice may be found in the fondness of the Chinese for mixing crops—a practice in operation all over the country.'

² Wallace in his chapter on 'Rotations and Mixed Crops' [8] wrote: 'The system is more or less known in this country [Britain], though not extensively followed!'

para. 60, Voelcker [8] remarked, apropos of the then recent discovery of nitrogen-fixation by legumes and their nodule bacteria:

India, to my mind, presents special advantages for the elucidation of the problem, one which, when solved, will unfold much that is still unexplained in the advantage of rotation of crops.

One of the most remarkable effects of the leguminous crop, whether in mixture or in rotation, is its apparent ability to supplement animal manures. In peninsular Indian practice (the best-studied case) it would seem that legumes grown in mixture can to a large extent fill the place of animal manures. It appears unlikely that this ability is due solely to the nutrient nitrogen compounds supplied by the legumes, and it is probable that leguminous plants everywhere make a definitely biological contribution to the fertility of soil.

REFERENCES

1. H. G. THORNTON and HUGH NICOL, The Effect of Sodium Nitrate on the Growth and Nitrogen-content of a Lucerne and Grass Mixture. *J. Agric. Sci.*, **24**, 1934, 269.
2. HUGH NICOL. The Derivation of the Nitrogen of Crop Plants, with special reference to Associated Growth. *Biol. Rev.*, **9**, 1934, 383.
3. H. G. THORNTON and HUGH NICOL, Further Evidence upon the Nitrogen-uptake of Grass grown with Lucerne. *J. Agric. Sci.*, **24**, 1934, 540.
4. H. H. MANN, D. L. SAHASRABUDDHE, N. V. KANITKAR, V. A. TAMHANE, and others, Land and Labour in a Deccan Village. Univ. Bombay Economic Series, No. 1. Oxford Univ. Press, London and Bombay, 1917.
5. H. H. MANN and N. V. KANITKAR, Land and Labour in a Deccan Village: Study No. 2. Univ. Bombay Economic Series, No. 3. Oxford Univ. Press, London and Bombay, 1921.
6. J. MOLLISON, A Text-Book on Indian Agriculture, vol. iii: Field and Garden Crops of the Bombay Presidency. Bombay, 1901.
7. — Imp. Gazetteer of India, vol. iii, chap. i (Agriculture). 1907 (as quoted in a letter from H. H. Mann).
8. J. A. VOELCKER, Report on the Improvement of Indian Agriculture. London, 1893.
9. R. WALLACE, India in 1887. Edinburgh, 1888.
10. A. HOWARD, Crop-production in India. Oxford Univ. Press, London, 1924.
11. H. KASERER, Beobachtungen über die Bewurzelung der Kulturpflanzen bei Reinsaat und bei Mischsaat. *Zeitschr. f. d. landw. Versuchsw. in Oesterreich*, **14**, 1911, 1022.
12. J. W. LEATHER, Final Report of the Agric. Chemist to the Govt. of India, 1897.
13. H. H. MANN, The Tea Soils of North-East India and their Treatment. Indian Tea Assoc., Calcutta, 1907.
14. F. R. IRVINE, A Text-Book of West African Agriculture. Oxford Univ. Press, London, 1934.
15. J. C. WILLIS, Agriculture in the Tropics. Univ. Press, Cambridge, 1914.
16. L. CARRIER, The Beginnings of Agriculture in America. New York and London, 1923.
17. THOMAS HARIOT, A Briefe and True Report of the New Found Land of Virginia. (Frankfort a. M., 1590.) Holbein Society edn., Manchester, 1888.
18. J. PINKERTON, Travels, vol. iii, p. 294. London, 1819 (as quoted by Carrier [16]).
19. J. SMITH, The Travels of Captaine John Smith, vol. i. Reprint, Glasgow, 1907.
20. I. HEDDE (Translation), Der Ackerbau in China. Leipzig, 1853.
21. R. FORTUNE, Visit to the Tea-districts of China and India. London, 1852.

(Received December 14, 1934)

THE INFLUENCE OF RAINFALL ON THE YIELD OF MANGOLDS AT ROTHAMSTED¹.

By R. J. KALAMKAR, Ph.D.

(*Statistical Department, Rothamsted Experimental Station,
Harpenden, Herts.*)

(With Two Text-figures.)

INTRODUCTION.

THE variation in the yield of mangolds on the twenty-five plots of the Barnfield mangold experiment at Rothamsted was discussed in an earlier paper. The variation was classified into three types: (a) deterioration owing to the exhaustion of plant nutrients in the soil; (b) slow changes other than deterioration, which are purely of a local nature, *e.g.* on the dunged plots they were attributed to the quality of the dung, the prevalence or otherwise of weeds, and the erosion of some of the plots due to surface flow of water; and (c) annual variation due to weather.

The object of the present paper is to study the influence of rainfall—one of the important weather elements—on the yield of mangolds.

METHOD.

The method employed in this investigation is essentially the same as that developed by Fisher(1). Instead of taking the rainfall for the whole year, a period from March 17 to November 17, both days inclusive, is chosen for the purpose. The rainfall is then divided into forty-one periods of 6 days each. Each set of forty-one values is then analysed by fitting a polynomial of the fifth degree. The amount and distribution of rain in each season is thus represented by a' , b' , c' , d' , e' and f' , the rainfall distribution constants which are then regarded as the six independent variates to be correlated with yield. The value of the rainfall distribution constants a' , b' , c' , d' , e' and f' for the fifty-five seasons 1876–1930, are given in the Appendix. For tabulation they have been multiplied by 1000.

The next step consists of fitting a polynomial of the fifth degree to

¹ Part of thesis approved for the degree of Ph.D. in the University of London, 1932.

572 *Influence of Rainfall on the Yield of Mangolds*

the set of fifty-five values of each variable with a view to eliminating any secular trend in each variate. The secular changes in the six rainfall constants are given in Table I.

Table I. *Secular changes in the rainfall constants.*

	a'	b'	c'	d'	e'	f'
Mean	482.33	40.38	3.49	1.04	2.05	0.11
x_2'	-123.45	-27.03	+26.00	-12.45	+4.14	-0.46
x_3'	+130.97	+9.52	-6.64	+11.95	-18.30	+48.70
x_4'	-111.68	+81.76	-4.13	+24.06	-1.35	-28.34
x_5'	-101.06	-5.30	+21.11	+12.90	-7.81	+14.19
x_6'	-3.17	-26.22	-1.36	+28.79	-23.84	-42.11
Standard deviation	99.78	48.09	39.86	33.69	24.59	14.79

It will be observed from Table I that no significant changes have taken place in the first five values of the rainfall constants. They fluctuate with large standard deviations about the mean values given in the first line of Table I. With f' , however, the mean appears to show the gradual change, the x_3' term being significant. The mean values c' , d' , e' and f' do not differ significantly from zero but b' differs significantly from zero. The course of the rainfall sequence during the season (March 17 to November 17) could therefore be represented by a straight line.

The crude sums of squares and products of the rainfall distribution values are then calculated from the figures given in the Appendix, and the sums of squares and products of residuals of these independent variates can now be obtained by applying the appropriate corrections calculated from Table I. These corrected sums of squares and products are given in Table II. The number of degrees of freedom in each case is 49, since out of the 54 degrees of freedom for the fifty-five seasons, 5 are already accounted for by the fitted terms of the polynomial.

Table II. *Sums of squares and products of deviations for the rainfall distribution constants.*

	a'	b'	c'	d'	e'	f'
a'	+487,882	+48,269	-50,586	-14,379	+2,512	-22,219
b'	+48,269	+113,327	+28,291	+168	-1,856	-5,211
c'	-50,586	+28,291	+77,857	+30,940	+10,150	-119
d'	-14,379	+168	+30,940	+55,618	-143	+549
e'	+2,512	-1,856	+10,150	-143	+29,612	+2,525
f'	-22,219	-5,211	-119	+549	+2,525	+10,713

We require to find the regression of the yield of mangolds for the different plots on each of these rainfall constants. It is therefore necessary to invert the above determinant so as to obtain a matrix of multipliers,

each of which is the co-factor of the number above, divided by the value of the determinant itself. Table III shows these multipliers in millionths.

Table III. *Co-factors for determining partial regressions.*

a'	b'	c'	d'	e'	f'
+2.768729	- 1.733834	+ 3.104890	- 1.064618	- 1.875972	+ 5.430241
- 1.733834	+ 11.416527	- 7.062233	+ 3.444913	+ 3.219529	+ 0.943385
+ 3.104890	- 7.062233	+ 23.576236	- 12.375674	- 9.367801	+ 6.108445
- 1.064618	+ 3.444913	- 12.375674	+ 24.621860	+ 4.930905	- 3.093808
- 1.875972	+ 3.219529	- 9.367801	+ 4.930905	+ 38.365510	- 11.724074
+ 5.430241	+ 0.943385	+ 6.108445	- 3.093808	- 11.724074	+ 108.055559

The secular changes in the yields of the twenty-five selected plots have already been considered in an earlier paper(2) and their values given in Table II of that paper. The course and extent of the changes are shown by the smooth curves drawn in Figs. 1-5 in the same paper. The annual deviation of the actual yield from the polynomial value forms the basis of the study of the effect of rainfall on the yield.

The sum of products of yield residuals and residuals of the six rainfall distribution constants, a' , b' , c' , d' , e' and f' , are now calculated for each of the twenty-five selected plots. The six products so obtained for each of the plots are multiplied by the values of any column of Table III and added, thus obtaining the regression of yield for the particular plot on

Table IV. *Partial regression of mangold yields on:*

	a'	b'	c'	d'	e'	f'
1 O	- 4.24101	+ 49.67809	- 43.28862	+ 36.88664	+ 57.15916	+ 35.20434
1 N	- 4.06640	+ 71.57131	- 63.29817	+ 57.16822	+ 85.23304	- 5.88600
1 A	+ 7.81353	+ 41.05541	- 37.69177	+ 32.26557	+ 71.33735	- 34.13797
1 AC	+ 18.67917	+ 32.66653	- 30.59509	+ 32.31197	+ 41.84961	- 33.41896
1 C	+ 8.89643	+ 43.86681	- 34.02688	+ 34.53737	+ 26.94570	+ 71.25084
4 O	- 3.80725	+ 7.69146	- 3.01634	+ 0.94171	+ 1.80040	- 6.60866
4 N	- 2.97953	+ 20.23670	- 16.37580	+ 43.27812	+ 43.80055	+ 8.89183
4 A	+ 2.32846	+ 22.48797	- 16.68334	+ 30.56133	+ 31.93497	+ 6.70498
4 AC	+ 10.48090	+ 34.21222	- 34.55675	+ 34.08437	+ 36.79476	- 6.49165
4 C	- 4.21198	+ 43.70662	- 36.06505	+ 22.36508	+ 54.34480	- 37.21136
5 O	- 4.01523	+ 9.14806	- 7.28551	+ 6.24444	+ 5.53394	+ 4.60130
5 N	- 5.87104	+ 13.77885	- 14.13987	+ 31.09841	+ 28.04198	- 57.73147
5 A	+ 8.36673	- 4.88590	- 5.70683	+ 19.25497	+ 9.14152	- 16.33541
5 AC	+ 21.13074	- 10.82524	- 18.14310	+ 21.42997	+ 7.69748	- 8.32391
5 C	+ 10.39144	+ 1.40830	- 15.46144	+ 11.85830	+ 21.55703	- 21.28773
6 O	- 2.47387	+ 1.84892	+ 1.91014	+ 1.65306	- 0.97300	+ 3.66984
6 N	- 5.32915	+ 28.30600	- 18.23361	+ 40.48992	+ 38.51469	+ 13.44461
6 A	+ 0.21427	+ 28.06673	- 25.35495	+ 39.27562	+ 30.16190	- 29.96958
6 AC	+ 6.75911	+ 29.29043	- 42.44667	+ 26.28500	+ 55.60687	- 34.15449
6 C	- 2.63581	+ 34.00453	- 30.80153	+ 13.86762	+ 43.61951	- 36.58791
8 O	- 5.07692	+ 7.50195	- 7.00959	+ 7.04084	+ 8.48139	- 2.26116
8 N	+ 3.00252	+ 0.35326	+ 1.32109	+ 33.68342	+ 5.31202	- 11.81593
8 A	+ 7.38212	- 4.57619	+ 6.13374	+ 17.48585	- 8.25019	+ 1.95945
8 AC	+ 9.82276	+ 4.42381	- 21.83256	+ 7.04590	+ 38.04608	- 8.49046
8 C	+ 6.77605	+ 9.03170	- 28.57634	+ 13.53637	+ 50.52664	- 13.93700

the corresponding rain variate. The values of the regression coefficients, the rainfall being measured in inches and the crop in tons per acre, are given in Table IV.

The totals for sets of 5 plots are given in Table IV A.

Table IV A.

	a'	b'	c'	d'	e'	f'
O	-19.61428	+ 75.86848	- 58.68992	+ 52.76669	+ 72.00189	+ 34.60566
N	-15.24360	+ 134.24612	- 110.72636	+ 205.71809	+ 200.90228	- 53.09696
A	+ 26.10511	+ 82.14802	- 79.30315	+ 138.84334	+ 134.32555	- 71.77853
AC	+ 66.87268	+ 89.76775	- 147.57417	+ 121.15721	+ 179.99480	- 90.87947
C	+ 19.21613	+ 132.01796	- 144.93124	+ 96.16474	+ 196.99368	- 37.77316
1	+ 27.08172	+ 238.83815	- 208.90053	+ 193.16977	+ 282.52486	+ 33.01225
4	+ 1.81060	+ 128.33497	- 106.69728	+ 131.23061	+ 168.67548	- 34.71486
5	+ 30.00264	+ 8.62407	- 60.73675	+ 89.88609	+ 71.97195	- 99.07722
6	- 3.46545	+ 121.51661	- 114.92662	+ 121.57122	+ 166.92997	- 83.59753
8	+ 21.90653	+ 16.73453	- 49.96366	+ 78.79238	+ 94.11594	- 34.54510

From these coefficients, the effect of an additional inch of rain at any time during the season on the yield of plots differently treated can now be calculated. For this purpose we divide the 6 regressions corresponding to any plot by factors of the form

$$\frac{(r!)^2}{(2r!)} 41.42 \dots (41 + r),$$

for A , B , C , etc., in the expansion of the average effect of rainfall in a series of orthogonal polynomials:

$$a = A + Bt + C(t^2 - n_2) + D\left(t^3 - \frac{n_4 t}{n_2}\right) + \text{etc.}$$

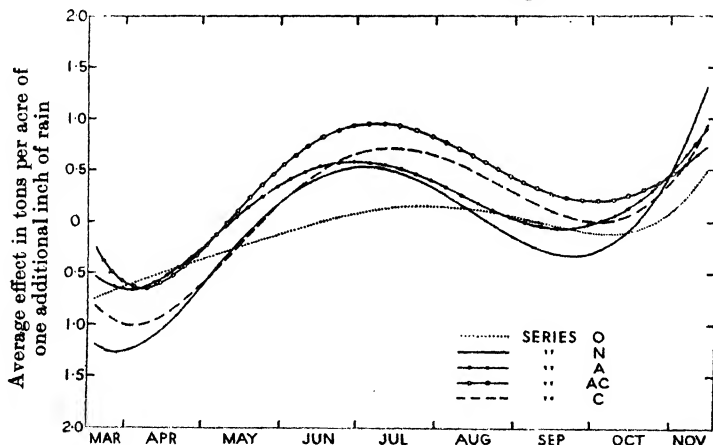


Fig. 1. Average curves for plots of series O, N, A, AC and C.

Series O, no nitrogen; N, nitrate of soda; A, sulphate of ammonia; AC, rape cake and sulphate of ammonia; C, rape cake.

(Fisher (1), pp. 97-99), where t is the time in 6-day intervals measured from the central period of the season, *i.e.* t runs from -20 to $+20$, n_2 is the mean value of t^2 and is equal to (n^2-1) , where $n=41$. The other

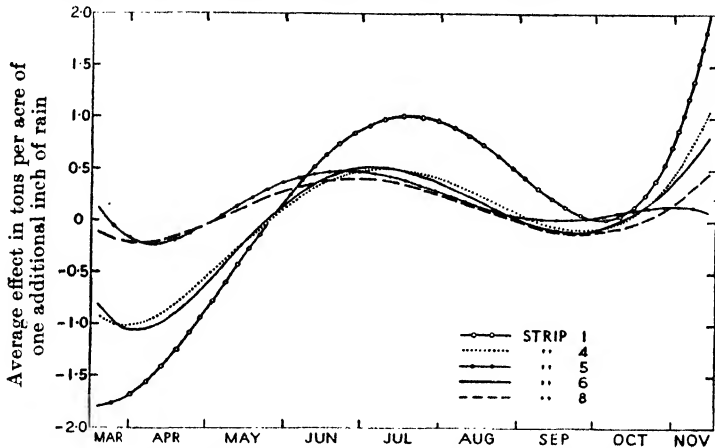


Fig. 2. Average curves for plots of strips 1, 4, 5, 6 and 8.
Strip 1, farmyard manure; 4, complete minerals; 5, super only;
6, super and sulphate of potash; 8, no minerals.

mean values, those of t^4 , t^6 , etc., are given by Fisher (1), p. 99). The values showing the average benefit or loss in tons per acre ascribable to an additional inch of rain at any time during the season, March 17 to November 17, for the differently manured plots, are plotted in Figs. 1 and 2.

ANALYSIS OF RESULTS.

The regression of yield on the six rainfall distribution constants are given in Table IV. It will now be of interest to see how the annual variation in the yield of mangolds is expressible in terms of the rainfall distribution constants. The mean squares due to the six regressions on the rainfall constants will be significantly greater than the mean square due to the deviations from the regression, in case the annual variation in the yield is to a large extent ascribable to the influence of rainfall. The result of such an analysis on plot 1 O is given in Table V. The sum of products of yield residual and residuals of a' , b' , c' , d' , e' and f' for plot 1 O are:

a'	b'	c'	d'	e'	f'
+1.3496	+3.9171	-0.0331	+0.7927	+1.2340	+0.3822

The regression of yield on the corresponding rain variates are:

a'	b'	c'	d'	e'	f'
-4.2410	+49.6781	-43.2886	+36.8866	+57.1592	+35.2043

576 *Influence of Rainfall on the Yield of Mangolds*

The sum of squares due to the regression on all the six variates is given by the sum of products of the regressions with the corresponding figures for the sum of products of yield residual and residuals of the rainfall constants, *i.e.*

$$[(+1.3496 \times -4.2410) + (+3.9171 \times +49.6781) + \dots].$$

The total sum of squares is the sum of squares due to annual variation after eliminating the secular changes in the yield. There are 6 degrees of freedom for the sum of squares due to regression, and 43 due to the deviations from the regression, making a total of 49 for the sum of squares due to annual variation.

Now, if a' had been the only measure of rainfall, the regression of yield on a' would be given by $1.3496 \div 0.4879$, *i.e.* $+2.7662$, where 1.3496 is the product of the yield residual and the residual of a' and 0.4879 is the sum of squares of deviations of a' values from their mean. The regression on a' alone is $+2.7662$ while the regression on a' when the distribution terms beyond a' are taken is -4.2410 . The total sum of squares due to the regressions with 6 degrees of freedom can be divided into two parts: (i) sum of squares due to a' only, with 1 degree of freedom, (ii) sum of squares due to the distribution terms b' , c' , d' , e' and f' with 5 degrees of freedom. Comparison of the mean square due to the distribution terms beyond a' with the mean square due to the deviations from the regressions will show the importance, if any, of the distribution terms beyond a' in adding to the value of the prediction of the yield. The result of the analysis on plot 1 O is given in Table V.

Table V. *Analysis of variance on plot 1 O.*

Variance due to	Degrees of freedom	Sum of squares	Mean square	$\frac{1}{2} \log_e$
Regression on a' only	1	3.7332	3.7332	—
Regression on b' , c' , d' , e' and f'	5	297.4104	59.4821	0.89152
Regression on a' to f'	6	301.1437	50.1906	0.80661
Deviations from the regression function	43	1111.7864	25.8555	0.47498
Total	49	1412.9301	28.8353	—

It will be observed that the distribution terms beyond a' have contributed to a large extent to the annual variation in yield. The value of z is 0.41654. It, however, just fails to reach the 5 per cent. level of significance. If a' were the only measure of rainfall it would not have given any information at all. The comparison of the mean square due to 6 degrees of freedom with that due to the deviation from the regression with 43 degrees of freedom shows that the weather element,

rain, accounts for only a small portion of the annual variation in the yield.

A similar analysis carried out on all the other plots shows that on none of them is the effect of rain significant. In fact, the mean square due to the deviations from the regression function with 43 degrees of freedom is slightly higher than that due to annual variation with 49 degrees of freedom on all the plots except the five dunged plots and 5 AC, 8 C and 8 AC. The cause for the annual variation in the yield of mangolds on the twenty-five plots studied cannot, therefore, be accounted for by the single weather element, rain.

Curves showing the average benefit or loss in tons per acre to be expected from an additional inch of rain over the average, at any time during the year, were prepared for all plots, but in view of the non-significance of the rainfall in accounting for the annual variation, curves are shown only for the means of five plots having each manurial ingredient, that is for the whole rows and columns of the plots as arranged on the field. All the plots except 5 AC, however, show in general an adverse effect of an additional inch of rain above the normal, up to the middle of May. During June and July, the additional inch of rain above the normal seems beneficial to the crop. On plots 5 A and 5 AC, however, there is no adverse effect of an excess of rain in the early part. The curves throughout are above the zero line, except in the case of 5 AC where it tends downwards towards the end.

The adverse effect of an additional inch of rain above the normal observed on most of the plots may partly be attributed to the difficulty in securing a proper tilth, which is a very important factor for the proper germination of the mangold seed, and also partly to the fact that rain during that period would delay the sowing and consequently shorten the period for the growth of the crop. The sowing period for mangolds extends from the middle of April to the middle of May, and the crop is ready for lifting by about the end of October or the first week of November. Rains during the months of June, July and August are beneficial for the rapid growth of the plant.

SUMMARY.

1. The influence of rainfall on the yield of mangolds is studied in this paper.
2. Analysis shows that the variation in the yield due to annual causes cannot be accounted for by a single weather element, rain.

3. An additional inch of rain above the normal, during the period extending from the middle of March to about the end of May, is harmful possibly because of the difficulty in securing a proper tilth and delay in sowing, which results in a shortening of the growing period.

4. The yield appears to be benefited by an additional inch of rain above the normal, particularly during the months of June and July.

Finally, it is with the greatest pleasure I thank Dr R. A. Fisher, F.R.S., of the Rothamsted Experimental Station, for his criticism in the preparation of this paper.

REFERENCES.

- (1) FISHER, R. A. The influence of rainfall on the yield of wheat at Rothamsted. *Phil. Trans. B* (1924), **213**, 89.
- (2) KALAMKAR, R. J. A statistical examination of the yield of mangolds from Barnfield at Rothamsted. *J. Agric. Sci.* (1933), **23**, 161.

APPENDIX.

Rainfall distribution constants (March 18 to Nov. 18 inclusive).

	a'	b'	c'	d'	e'	f'
1876	472	49	10	-35	-6	42
1877	509	12	40	19	29	14
1878	621	15	42	59	-15	39
1879	670	-53	-129	-2	17	6
1880	616	135	-7	-32	-5	6
1881	446	90	-38	-32	-5	14
1882	641	73	55	47	-31	-21
1883	481	60	-19	2	-3	-12
1884	345	26	-24	-3	-6	5
1885	526	47	19	-1	-58	-17
1886	496	29	50	36	-7	-11
1887	340	28	35	-4	-11	1
1888	543	1	-23	25	56	-5
1889	596	-14	-50	16	-6	-20
1890	463	0	-1	-6	54	-16
1891	572	112	11	-1	-24	-16
1892	484	105	-19	1	8	-10
1893	357	101	-25	-7	-3	-13
1894	546	151	53	73	39	35
1895	492	84	-5	-11	55	24
1896	579	113	14	-110	-9	-9
1897	371	-19	-23	-18	1	8
1898	327	-8	9	18	-18	-18
1899	485	82	60	28	-32	-14
1900	391	46	11	5	30	3
1901	364	-14	17	-24	6	19
1902	376	-2	-39	9	0	-6
1903	716	47	-46	-15	-2	-36
1904	355	18	-5	-6	8	14
1905	463	35	-9	15	12	2
1906	449	126	60	36	2	-24
1907	449	11	-2	20	-28	-14
1908	449	-49	4	-15	-16	10
1909	560	25	2	-2	0	-29
1910	424	38	-21	25	5	-4
1911	445	39	54	47	-4	-18
1912	572	57	-37	-52	45	-17
1913	428	20	69	14	-12	7
1914	345	19	36	6	16	-16
1915	508	56	-3	21	46	0
1916	548	38	60	-21	17	-23
1917	639	74	-68	-55	9	7
1918	555	9	12	-26	-45	21
1919	401	-27	8	-21	13	23
1920	461	-56	-13	-6	12	17
1921	259	33	33	-8	-15	15
1922	471	-15	-30	-11	7	18
1923	475	106	6	-14	-10	-4
1924	665	32	-3	24	-29	1
1925	500	84	-10	-22	-2	5
1926	499	65	40	76	9	14
1927	592	31	-44	-54	26	-1
1928	418	47	28	8	18	-5
1929	354	87	68	52	-8	0
1930	419	22	-21	-11	-17	15

(Received July 5th, 1933.)

[FROM THE ANNALS OF APPLIED BIOLOGY, Vol. XXI, No. 3,
pp. 398-417, August, 1934.]
[*All rights reserved.*]

THE EFFECT OF RUBIDIUM SULPHATE AND PALLADIUM CHLORIDE ON THE GROWTH OF PLANTS

BY WINIFRED E. BRENCHELEY, D.Sc.

(*From the Botanical Department, Rothamsted Experimental Station,
Harpenden, Herts.*)

(With Plate XX and 7 Text-figures.)

THE complexity of the factors bearing on plant growth has long been recognised, and problems relating to the effect of environmental conditions and the importance of various elements in the nutrition of the plant have engaged the attention of investigators for many years past. More recently it has become increasingly apparent that the elements concerned in the well-being of plants are more numerous than was originally realised, and that very small quantities of certain elements may be as vitally necessary as the larger amounts of nitrogen, phosphorus, potassium and others which have long been known to be essential. The poisonous nature of compounds of copper, aluminium, zinc, etc., has been recognised and in some cases turned to account, as in the use of Bordeaux mixture for protecting potatoes against attacks of *Phytophthora*, and of copper sulphate for the destruction of charlock amongst crops. It is also well known that the presence of large proportions of certain elements as zinc in soils is inimical to plant life, though some species possess a measure of immunity and flourish in such situations(24). On the other hand, small quantities of various compounds are credited with exercising a stimulating action on growth, though much of the evidence is conflicting owing to fundamental differences in the way the experiments have been carried out.

Quite apart from stimulation it is fully accepted that small amounts of boron(11,15) and manganese(20) are essential for the full development of many plants, and similar claims have been made for various other elements. It is quite within the bounds of possibility that some of the many obscure plant diseases or growth failures may prove to be due to the presence or absence of some element of whose importance we have at present no knowledge. Possibly, too, an element essential to one plant

may not be the same as that necessary for another, though this is at present entirely in the region of hypothesis. The available information is in reality very scanty, as comparatively little work has yet been done on many of the less plentiful elements occurring in various soils. Where such an element as rubidium is known to occur in appreciable quantity, information as to its action on plant growth might be of direct importance from an agricultural standpoint, and, as a matter of fact, enquiries with regard to this element have already been received.

From another point of view these less common elements need consideration. From time to time in the development of mining and industrial concerns, stores of certain elements tend to accumulate as by-products, for which a profitable commercial outlet is sought. Or, a particular element may be found as a constituent of fertilisers (as vanadium in some basic slags), when its action on plant growth becomes a matter of considerable economic importance. It is very desirable, therefore, that our information should be increased by work on as many elements as possible, even though the immediate practical application of the results may not be apparent. For this reason, therefore, rubidium and palladium have been examined with regard to their action on various plants, rubidium being specially chosen because of its close relation to potassium.

Water culture experiments with rubidium and palladium salts were carried out at Rothamsted in 1933, cereals being started in February, followed by leguminous crops in August. Pedigree seeds of barley (Spratt Archer), wheat (Little Joss), and oats (Starr) were provided by the courtesy of the National Institute of Agricultural Botany, Cambridge, and grown at first in a modification of the usual Rothamsted solution, pH 5.0, with half the usual amount of calcium. Later on in the summer, when the weather was very hot, all the phosphate was supplied as potassium di-hydrogen phosphate, giving a solution of about pH 3.8, which allows more healthy growth in cereals under sultry greenhouse conditions. Peas (Sutton's Pioneer) were grown in the same acid solution, but with the usual full supply of calcium, while the broad beans (Sutton's Prolific Longpod) were provided with one in which the mono- and di-hydrogen phosphates were adjusted to give a pH 6.2. These later variations were made to provide solutions known to be favourable either to growth conditions or the plants concerned (see table on p. 400).

In order to provide some definite means whereby, if necessary, the action of one element can be compared with that of another, molar solutions were taken as a basis. As these take into consideration the

	Cereals		Peas gm.	Beans , gm.
	Early gm.	Late gm.		
Potassium nitrate	1.0	1.0	1.0	1.0
Magnesium sulphate	0.5	0.5	0.5	0.5
Sodium chloride	0.5	0.5	0.5	0.5
Calcium sulphate	0.25	0.25	0.5	0.5
Potassium di-hydrogen phosphate	0.48	0.5	0.5	0.38
Potassium mono-hydrogen phosphate	0.108	—	—	0.27
Ferric chloride	0.04	0.04	0.04	0.04
Manganese sulphate	—*	0.001	0.001	0.001
Boric acid	0.001	0.001	0.001	0.001
Water (distilled)	To make up 1 litre			

* Manganese sulphate was not given at first but was added later, as the oats in particular began to show signs of deficiency.

valency of the elements, they provide a better basis of comparison than that of equivalence according to the atomic weight of the element. It was decided that in all cases a standard concentration of 1 c.c. $M/100$ per litre nutrient solution should be tested, other concentrations ranging above and below this as required. For convenience of reference this solution of $M/10^5$ is designated throughout as k .

The palladium as received was in the form of chloride, known to be a mixture of $PdCl_2$ and $PdCl_2 \cdot 2H_2O$, so the material was dissolved and the palladium estimated, the requisite calculations then being made on the amount of the metal present. As earlier tests had shown palladium to be definitely toxic, a range of $k/32 \times 2$ to $2k$ was adopted for cereals (with $4k$ for barley in addition), and modified to $k/16 \times 4$ to $4k$ for peas and beans tested against controls without palladium.

Rubidium was used in the form of sulphate. In this case a wider range of concentrations was adopted, $k/125 \times 5$ to $125k$ for cereals, changed to $k \times 10$ to $100k$ for peas and beans, tested against controls. In all cases each individual species was examined with both elements rendering it possible to compare the response from the basis of the $M/10^5 = k$ solution.

RUBIDIUM.

Rubidium is known to occur in some soils in very small amounts (26,31) and it has also been found in various plants, special methods being necessary for its quantitative determination in the presence of an excess of potash (21,27,33). As early as 1862 Grandeau (14) had determined the presence of rubidium in beetroot, tobacco, raisins and notably in coffee and tea, and concluded that the element was taken up from the soil and was probably widespread in the vegetable kingdom.

Pfeiffer^(22,23) analysed beet ash in northern France, obtaining about 1.75 gm. of rubidium chloride per kilogram of ash, from which he calculated that 255 gm. of rubidium chloride were taken out per hectare with every crop of beetroot. The ratios of the rubidium chloride to sodium and potassium chloride were as 1 : 126 and 1 : 331. Lippmann⁽¹⁶⁾ again verified the presence of the element in beetroot. More recently Robinson, Steinkoenig and Miller⁽²⁸⁾ determined the presence of rubidium in many plants, and found that it was not always a constant constituent of any one species, as it might be entirely absent, or present in appreciable amounts in different samples of material. They concluded that rubidium was absorbed by plants if it is present in the soil solution, but they obtained no evidence of any fertilising value.

The physiological importance of rubidium is not very clear. In spite of the close relationship between the two elements, rubidium cannot replace potassium in the metabolism of the plant. Loew⁽¹⁷⁾ found that though rubidium chloride permitted normal growth of buckwheat up to flowering time, changes in the chlorophyll, accumulation of sugar and various hindrances to normal metabolism then occurred and gradually killed the plant. Rubidium nitrate caused starch accumulation, resulting in thickening and twisting of the stem, with curling and fleshiness of the leaves, and the plants died before the production of flowers. Later on Loew⁽¹⁸⁾ expressed his belief that rubidium can replace potassium in the nutrition of some of the lower fungi. On the other hand, in the complete absence of potassium *Aspergillus niger* produces no spores in the presence of rubidium chloride^(29,32) and the total weight of the mould is reduced, but the addition of potassium at a later stage results in spore production. Arndt⁽³⁾ also found that buckwheat grown with rubidium salts instead of potassium showed definite signs of injury, such as premature dying of the roots, curling of leaves and bleaching of chlorophyll. The symptoms were allayed or removed by the addition of potassium salts, and it was inferred that the injurious action of the rubidium salts was due solely to a disturbance of metabolism, and that antagonism exists between potassium and rubidium if both are available together.

More recent work⁽⁴⁾ with oats suggests that rubidium interferes with potash assimilation even though it may have no specific toxic effect. Alten and Gottwick⁽¹⁾ state that the gradual substitution of rubidium for potassium was deleterious to the growth of oats and tobacco in soil cultures, causing decreased yield and depression in various ash constituents, the harmful effect being attributed to disturbances in the equilibrium of the cell contents. The dry weight of tobacco was reduced

402 *Effect of Rubidium Sulphate etc. on the Growth of Plants*

by the presence of as little as 0.009 per cent. rubidium oxide in the soil. Attempts to replace potassium by rubidium for maize and barley grown in water cultures were unsuccessful⁽³⁰⁾, the yields falling gradually to zero as the proportion of rubidium to potassium increased.

Another aspect of the physiological action of rubidium is that of stimulation. Rubidium salts at 0.2 per cent. concentration are said to hasten the germination of barley⁽⁶⁾, and the production of roots on cut stems of *Alternanthera spathulata* has been stimulated⁽²⁾. While low concentrations of rubidium salts may improve growth^(5,13,19,25,34) it is usually found that greater strengths cause damage and retard growth even if they do not kill the plants. A characteristic feature noticed by various workers in the latter cases is checking of root growth, often associated with swelling of the roots just behind the tip, recorded by Bokorny for peas, Wolkenhauer for oats and Alten and Gottwick for tobacco.

The 1933 Rothamsted experiments were designed to determine the action of a wide range of concentrations of rubidium sulphate on various plants in the presence of an adequate supply of normal nutrients including potassium. The aim was not to determine the toxicity of large amounts of rubidium, but to examine the possibility of improvement in growth.

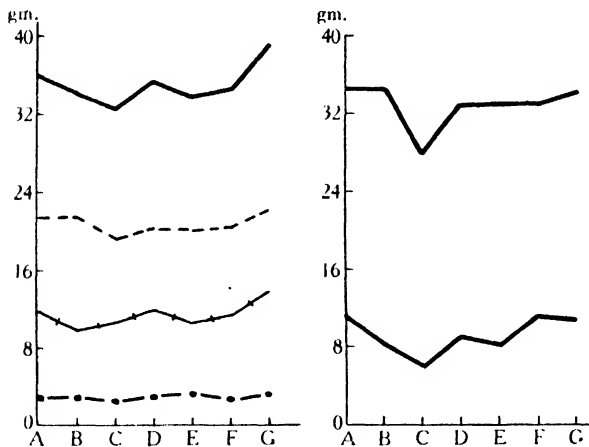
All the cereal seeds were graded in weight, sown on February 17th in clean moist sawdust, and transferred to nutrient solutions on March 1st. After the first five weeks the solutions were changed fortnightly, with fresh supplies of the test substances, until harvest (barley July 20th, oats July 21st, wheat August 2nd). The plants were randomised in five blocks containing one replicate of each concentration, to avoid growth differences due to position which would prevent statistical estimation of the standard error. Barley made excellent growth, but with wheat the choice of the variety Little Joss proved to be unfortunate, as the plants developed a dense prostrate habit, forming large numbers of tillers of which very few eventually grew erect and produced belated ears. In the field Little Joss may be sown either in autumn or spring, but if sowing is delayed after February there is a tendency towards a similar habit. Apparently the condition of growth in water culture encouraged this abnormal development in spite of early sowing, and the response to rubidium can only be considered in relation to vegetative growth, as very little grain was produced. The oats also failed to come up to standard in their development, and ripened hardly any grain. The cause of this is not clear, but may be associated with the type of nutrient

solution, the omission of manganese sulphate during the earlier weeks of growth, or with the incidence of fungal attack on the seed coats soon after the seedlings were placed in solution. The general trend of events, however, was obviously the same as in wheat and barley, and the dry weight curve is inserted for the sake of comparison.

Table I.

Effect of rubidium sulphate on the growth of barley
(means of five replicates).

Treatment ($k = M/10^5$)	No. of tillers	No. of ears	Dry weight			
			Shoot gm.	Root gm.	Grain gm.	Total gm.
A. Control	16.4	14.8	21.33	2.93	11.69	35.95
B. $k/125$ Rb_2SO_4	17.0	14.8	21.40	2.93	9.77	34.10
C. $k/25$ "	14.4	13.8	19.38	2.51	10.69	32.58
D. $k/5$ "	14.4	14.0	20.38	3.09	11.90	35.37
E. k "	17.2	13.4	20.13	3.23	10.33	33.69
F. $5k$ "	16.6	15.6	20.43	2.66	11.23	34.32
G. $25k$ "	17.4	16.2	22.09	2.95	13.88	38.92
		S.E.	0.98	0.22	1.019	1.94



Text-fig. 1. Dry weights of cereals grown with rubidium sulphate. Left, barley; right, wheat (upper), oats (lower). — total; - - - shoot; -+- grain; ····· root. (For concentrations A-G see Table I.)

With all these cereals no variation was observed during growth at any stage of development which could be attributed to any concentration of rubidium sulphate. In barley all root and shoot growth was normal and similar to that of the control plants, ear emergence occurred simultaneously throughout the series with no significant difference in the number of ears, and the ripened grain was all fit to harvest at the

404 *Effect of Rubidium Sulphate etc. on the Growth of Plants*

same date (Plate XX, fig. 1). With wheat and oats, also, the plants receiving rubidium sulphate developed in just the same way as the controls. These visual observations were fully borne out by the dry weights, the variation between individual plants with the same treatment or in the same randomised blocks being as great as that caused by treatment with different concentrations of rubidium sulphate, the standard errors of the results of treatment being insignificant (Table I and Text-fig. 1).

In the above experiments week old seedlings were used which had not been treated with rubidium during germination. In case this immune period might have affected the after results, barley seeds were soaked for six hours on November 30th in rubidium sulphate solutions of the same graded strengths as in the main experiment. These seeds were then germinated in beakers on perforated slabs of paraffin wax floating on 100 c.c. of similar rubidium sulphate solutions in two series, one with distilled water and the other with complete nutrient solution. Satisfactory growth was made and after six weeks there was no obvious difference in the growth of the seedlings due to increasing strengths of rubidium sulphate, thus corroborating the previous results. The method was not satisfactory with wheat, peas and beans, owing to the rapid growth of moulds on the germinating seeds.

Water culture experiments carried out in autumn with peas gave similar results to those with cereals, no significant differences due to the rubidium sulphate being observed (Table II and Text-fig. 2).

With broad beans there was an increased dry weight with the two lower concentrations which approached significance, especially in the comparison between the controls and those receiving 10*k* rubidium sulphate. As beans are so extremely variable in growth, little weight can be placed on this one instance of suggested improvement.

The general indication from these experiments is that in the presence of adequate supplies of potash, rubidium sulphate in concentrations of 125*k* = (125 *M*/10⁵) and less has no appreciable influence on growth. The maximum amount of rubidium sulphate used represented 0.01668 per cent. by weight, other workers having reported beneficial effects with much heavier doses up to 0.2 per cent. (18), and, using the chloride, with as little as 10 mg. to 50 kg. soil, equivalent to 0.00002 per cent. (23).

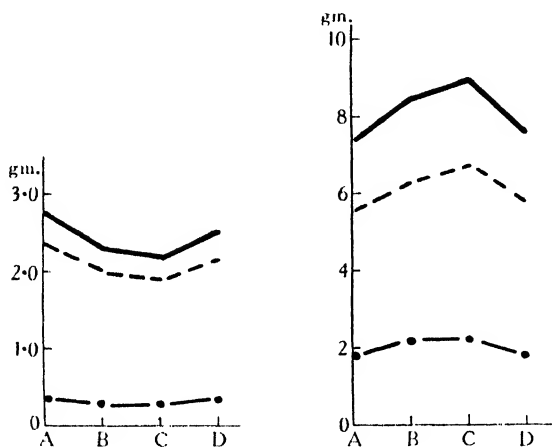
It would seem to be fully established that rubidium cannot replace potassium in the plant economy, but in view of the great discrepancies in the results of various workers it has not yet been definitely shown that, *in the presence of adequate supplies of potash*, large doses of rubidium

salts are definitely toxic, or that very small quantities exercise a stimulating action on growth.

Table II.

Effect of rubidium sulphate on the dry weight of peas and broad beans (means of five replicates).

Treatment ($k = M/10^3$)	Peas			Broad beans		
	Shoot gm.	Root gm.	Total gm.	Shoot gm.	Root gm.	Total gm.
A. Control	2.37	0.38	2.75	5.57	1.79	7.36
B. k	2.01	0.28	2.29	6.24	2.22	8.46
C. $10k$	1.90	0.27	2.17	6.72	2.24	8.96
D. $100k$	2.15	0.35	2.50	5.75	1.80	7.55
S.E.	0.33	0.07	0.38	0.41	0.19	0.55



Text-fig. 2. Dry weights of peas (left) and broad beans (right) grown with rubidium sulphate. — total; - - - shoot; ····· root. (For concentrations A-D see Table II.)

PALLADIUM.

Very little work has been done with palladium in connection with plant growth, as it is not one of the rarer elements which is widespread in soils and its presence in plants does not seem to have been recorded. Coupin (12) found that palladium chloride was very strongly toxic to wheat in the early stages of germination, and its toxicity to broad beans was found some years ago in Rothamsted experiments, in an attempt to replace boron by palladium in culture solutions. Its behaviour in these preliminary tests suggested the recent trials with a range of concentrations and various species of plants, as indicated in the introduction.

Barley. The series in general was very strong and healthy, and the action of the increasing concentrations of palladium showed such a marked gradation that it is advisable to set out in some detail the observations on the developmental history of the plants (Plate XX, fig. 2).

Throughout growth concentrations of $k/16$ and less had no apparent influence on growth, either adverse or beneficial, and this was corroborated by the yield figures. All higher concentrations checked development from the first, the initial degree of checking becoming more marked with increasing concentration. With $k/8$ root growth was hindered and tillering was delayed, but after about six weeks growth proceeded more rapidly and after ten weeks the plants had caught up to the controls. No further advance was made beyond the controls, and yields were parallel in both cases.

With twice this strength of palladium $k/4$ the initial check was much more severe, and recovery considerably later. At an early stage the deficient functioning of the roots was shown by a reddening of the shoots, a very usual indication of starvation which can occur in the midst of plenty if absorption is hindered. Eventually improvement occurred, though the plants throughout appeared weaker than the controls.

From this concentration upwards the toxic effect of palladium was much more definite, and distinct morphological differences were manifested. The initial check was almost complete, the red colour of the stem was very marked, and when growth did occur the development was abnormal. With $k/2$ the roots grew very slowly and were short, thick and stubby in nature, evincing an apparent reluctance to enter the solution. Here again improvement set in after about six weeks. Longer and thinner roots grew out, tillering began, and the plants slowly assumed a more normal and characteristic appearance, but the progress of ripening was delayed and the ears did not change colour so early as those of the controls.

With k the primary check was more severe and lasted longer before progress began. The tips of the stubby roots showed curious thickenings, recalling those mentioned by Wolkenhauer with rubidium (21). These were quite firm in texture, unlike the flabby thickenings which sometimes appear when roots come in contact with toxic solutions. Tillering was considerably delayed, as neither shoots nor roots made any definite headway for more than six weeks after the experiment was set up, and the ultimate yield was much reduced, while the grain was still green at the time of harvest.

With $2k$ the same thickenings occurred at the root tips, but at first

it appeared that no recovery would be made. However, between the sixth and tenth week amazing progress occurred, a good number of tillers were produced and fairly good roots put forth, though the latter were thicker than normal, after which growth proceeded steadily and a comparatively good yield resulted. The effect of $4k$ concentration was in some respects the most surprising of all. The initial check was so severe that the plants seemed almost dead; no root movement occurred at all, and the shoot remained practically undeveloped. So bad seemed the condition that the discarding of the plants was considered. This state of utter stagnation continued for over six weeks, after which signs of renewed vitality became manifest. A month later some degree of root development had occurred in several plants, coupled with the production of a few tillers. These plants remained very small, but most of them developed one or more earing shoots, though grain production was very feeble and the ears were quite green at harvest.

This recovery in strong concentrations of inorganic poisons was unexpected. When working with other elements, as copper, zinc, arsenic, etc., it had usually been found that if the toxic compound is sufficiently concentrated to cause severe check to growth, no ultimate recovery occurs if this primary check is at all prolonged (7, 8). Recovery with gold and mercury was, however, observed in broad beans, but the period of stagnation was much shorter (11). On the other hand, with organic compounds as hydrocyanic acid (9) and phenols (10) some measure of recovery frequently occurs after a dormant period of a month or more, particularly when the nutrient solutions are not changed too often, provided the poison is not so strong as to kill the plant immediately. This had been attributed to a decomposition of the organic compounds into less harmful substances, but in the case of inorganic salts a similar disorganisation of the toxic substance does not occur, as the poisonous property is associated with the element itself. A possible explanation of the behaviour of barley in strong palladium chloride solution may lie in the change in climatic conditions during growth. The summer of 1933 was unusually hot and sunny, and a definite and rather sudden rise in the maximum and mean temperatures occurred towards the end of April, when the maximum was frequently 80° F. instead of 70° F. as was usual in the earlier part of the experiment. This increase in temperature coincided rather closely with the general impaired growth of the barley which was adversely affected by palladium, and may have provided the impetus which enabled even the most badly checked plant in $4k$ to produce at least a modicum of normally developed roots and shoots.

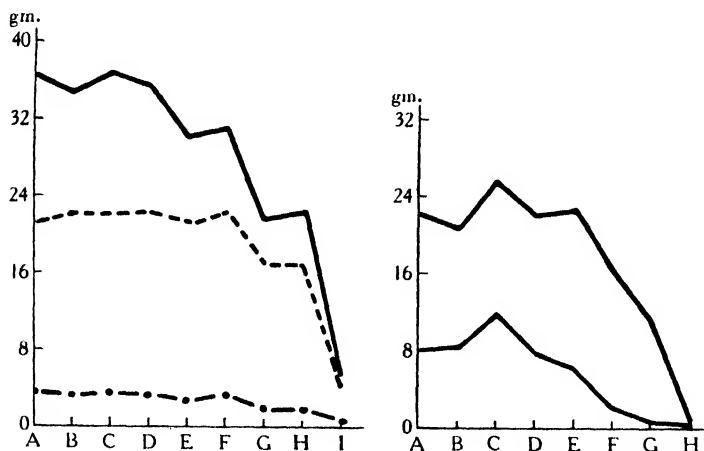
408 *Effect of Rubidium Sulphate etc. on the Growth of Plants*

The plants were harvested and dried when the controls had ripened their grain. The three weakest concentrations had no effect on the dry weight, but with stronger solutions a depression set in, becoming more marked with increasing palladium until with $4k$ the total dry weight was only 5.3 gm., representing but 14.4 per cent. of that of the control (Table III and Text-fig. 3).

Table III.

Effect of palladium chloride on barley (means of five replicates).

Concentration ($k = M/10^5$)	Dry weight				No. of tillers	No. of ears
	Shoot gm.	Root gm.	Grain gm.	Total gm.		
A. Control	21.23	3.56	11.92	36.71	17	15
B. $k/32$	22.07	3.27	9.47	34.81	17	16
C. $k/16$	22.01	3.45	11.35	36.81	21	16
D. $k/8$	22.16	3.22	10.05	35.43	15	15
E. $k/4$	21.23	2.59	6.06	29.88	17	15
F. $k/2$	22.15	2.93	5.97	31.05	25	15
G. k	17.13	1.83	2.42	21.38	22	14
H. $2k$	16.68	1.85	3.71	22.24	17	13
I. $4k$	4.41	0.66	0.23	5.30	6	3
s.e. excluding $4k$	1.67	0.30	1.26	2.96		



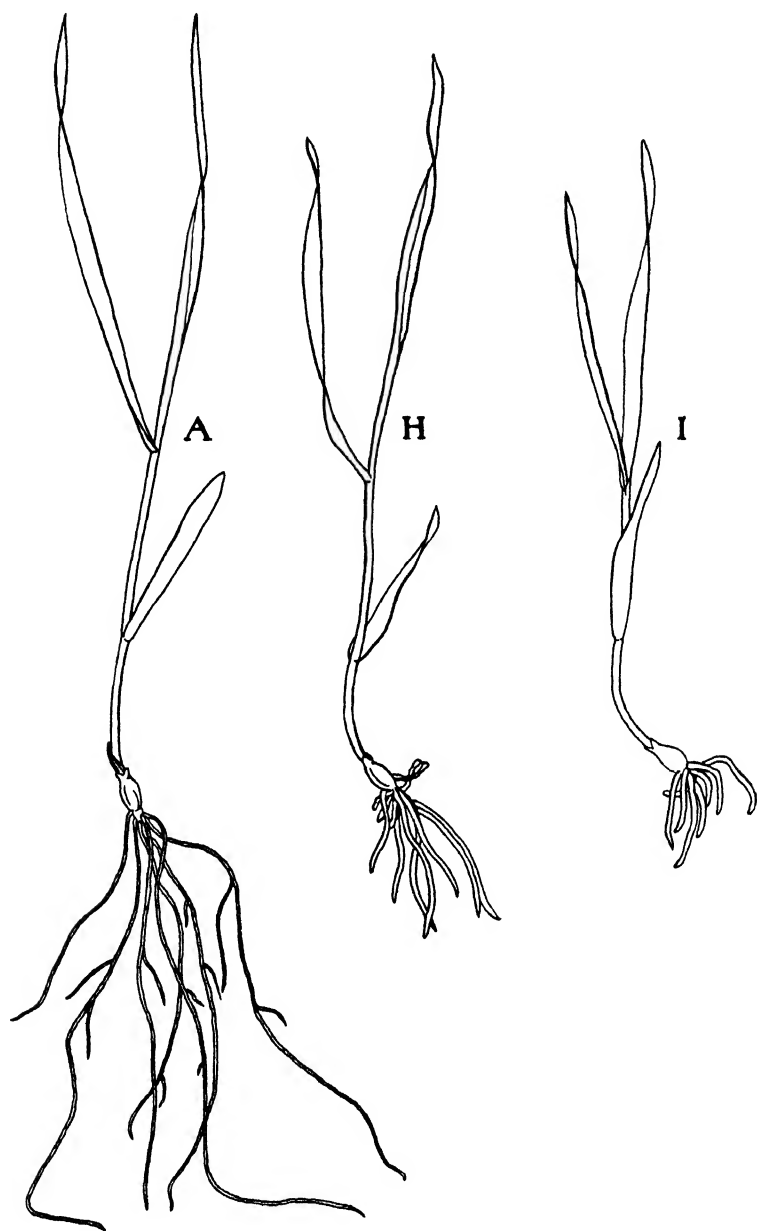
Text-fig. 3. Dry weights of cereals grown with palladium chloride. Left, barley; right, wheat (upper), oats (lower). — total; - - - shoot; · · · · root. (For concentrations A-I see Table III.)

The response of the different plant organs, however, was not the same, some being more influenced than others. The roots showed certain variable fluctuations in the weaker strengths, but no significant drop was indicated until k was reached, and though the shoots appeared to

respond at the same point statistical examination of the individual results show that the differences were not significant, except for the sudden drop with $4k$. Actually, however, a definite adverse action was evident in spite of the non-significance of the dry-weight figures from one concentration to another. The indicated drop in the k and $2k$ shoots was due to certain plants which failed to recover from the initial check so completely as their fellows, suggesting that at about these concentrations the degree of shoot recovery depends upon the inherent vigour of the individual plants. It is tempting, also, to attribute the heavy tillering with $k/2$ and k to the action of the poison, but in view of a corresponding increase with $k/16$ it is unsafe to make this assumption. The total number of ears was not significantly affected except with the strongest concentration. The grain showed much more definite reaction to the palladium, significant decrease in dry weight setting in with $k/4$, after which the decreasing weight with increasing concentration was associated with a corresponding delay in ripening, many of the ears being still quite green when the controls were dead ripe. Germination tests on barley were carried out as with rubidium. During the first week the amount of growth decreased steadily as the palladium solutions became stronger, from the lowest concentrations upwards. The less checked plants gradually caught up the controls, but with the strongest concentration this recovery did not occur during the six weeks of the experiment, and the roots with $2k$ and $4k$ made very little growth (Text-fig. 4). The general trend of events was similar in both series, but in the absence of nutrient salts the weaker concentrations of palladium chloride were somewhat more toxic and recovery was not so complete.

Wheat. In the early stages the weakest concentration which checked growth was $k/8$ as in barley, the set-back becoming more marked with increasing palladium until with k and $2k$ there was practically no development. With the weaker concentrations the degree of recovery with time was very parallel to that in barley, but with greater strengths wheat did not make such good progress, and with the strongest concentration very little growth occurred throughout (Text-fig. 3), whereas under similar conditions barley plants produced about half as much dry matter as the controls.

Reference has already been made to the abnormal character of the growth of wheat. Tillering was very heavy, but when the experiment was concluded the numerous early tillers had died down leaving the few green shoots which carried unripe ears. At this stage no check was evident with $k/4$, but as the palladium increased to $2k$ the harmful action



Text-fig. 4. Effect of palladium chloride on the germination and early growth of barley in nutrient solutions. (For concentrations A, H, I see Table III.)

became very strongly marked in total dry weight, number of tillers and ear production. The thickening and stubbiness of roots, characteristic of poisoning, was manifest in all the checked plants.

Oats. Oats gave signs of being more sensitive than either wheat or barley to the harmful action of palladium, as growth was almost entirely



Text-fig. 5. Effect of palladium chloride on peas, after eight days in solution. A, control; E, with strong solution. (For concentrations see Table IV.)

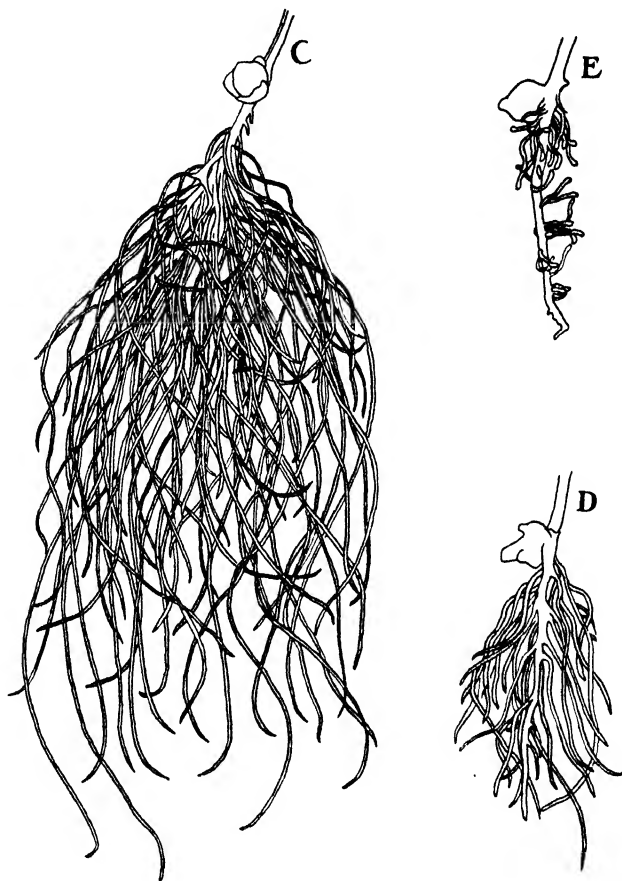
checked at concentrations which allowed good growth in the other cereals. The development, however, was very poor, and it is possible that this prevented the recovery from toxic effects which might have occurred in stronger plants.

The initial checking was again evident with $k/8$, progressive injury occurring with increasing concentration (Plate XX, fig. 3). Abundant root-hairs were a noticeable feature of the most strongly poisoned plants.

412 *Effect of Rubidium Sulphate etc. on the Growth of Plants*

As time went on stubby roots were manifest from $k/4$ upwards, being more marked at lower concentrations than in wheat or barley.

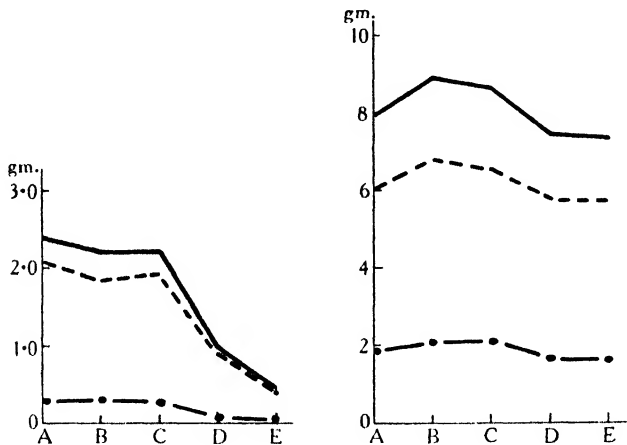
By harvest time all the plants had developed a certain number of panicles, which had changed colour and ripened off, but hardly a fertile grain was produced, even the controls having none.



Text-fig. 6. Effect of palladium chloride on peas, after seven weeks in solution. C showing complete, and D partial, recovery from initial check. (For concentrations see Table IV.)

Peas. Peas were grown in late summer and showed the great toxicity of the higher concentrations of palladium. From the first the heaviest dose ($4k$) drastically checked the roots which became yellow and produced stubby knotted laterals (Text-figs. 5 E and 6 E), the shoots being correspondingly small. In this case there was no recovery as time went

on. With one quarter the strength (k) a certain amount of lateral root growth ultimately occurred (Text-fig. 6 D), but the plants were seriously damaged, the shoots being small and the roots yellow. With the weaker concentrations, however, the initial check was completely overcome (Text-fig. 6 C) and the final development was as good as that of the control plants receiving no palladium. When harvested after two months' growth the roots in the two strongest concentrations were still stunted and very dark or black, the shoots being small and dying. Even the most badly poisoned plants, however, had flowered and were forming pods. The drop in dry weight was sharper in the shoot than in the roots (Table IV, Text-fig. 7), and the total production with $4k$ palladium was only about 20 per cent. of that of the controls.



Text-fig. 7. Dry weights of peas (left) and broad beans (right) grown with palladium chloride. — total; --- shoot; root. (For concentrations A-E see Table IV.)

Table IV.

Effect of palladium chloride on the dry weight of peas and broad beans (means of five replicates).

Treatment ($k = M/10^5$)	Peas			Broad beans		
	Shoot gm.	Root gm.	Total gm.	Shoot gm.	Root gm.	Total gm.
A. Control	2.08	0.31	2.39	6.04	1.86	7.90
B. $k/16$	1.85	0.37	2.22	6.80	2.09	8.89
C. $k/4$	1.93	0.28	2.21	6.56	2.11	8.67
D. k	0.92	0.07	0.99	5.77	1.68	7.45
E. $4k$	0.39	0.04	0.43	5.72	1.61	7.33
S.E.	0.21	0.045	0.27	0.53	0.16	0.67

414 *Effect of Rubidium Sulphate etc. on the Growth of Plants*

Broad beans. This was the only species in which the toxic action of the stronger concentration of palladium was not very noticeable. In the early stages the plumules and roots were somewhat stunted with heavy doses of palladium. The shoots made a complete recovery and developed normally. Some thickening and shortening of the roots persisted throughout growth, but was not very marked. The dry weights showed no significant differences due to treatment, the variation between individuals being greater than that due to palladium (Table IV, Text-fig. 7).

Summing up the general results, as far as the three cereals are concerned the action of palladium appears to be very similar, with certain differences in the range at which the toxic action is manifest. In all cases an early check with comparatively low concentrations occurs, followed by a gradual recovery, which does not necessarily affect all the plant organs in the same degree, the roots and grain, in barley at least, being more influenced than the shoot. The poisoned roots become thick and stubby, entering the solution with reluctance, but later in life considerable recovery towards normal occurs, such recovery being possible in barley in the presence of higher concentrations of palladium than with either wheat or oats.

SUMMARY.

1. Comparisons have been made between the action of rubidium sulphate and palladium chloride on the growth of barley, wheat, oats peas and beans in complete nutrient solutions.

2. Over a wide range of concentrations *rubidium sulphate* was not found to exercise either a beneficial or a harmful action on the growth of any of the species tested. The germination of the seeds was likewise not affected.

3. No benefit was derived from *palladium chloride*, but at a comparatively low concentration a harmful action occurred which became more intense with increasing concentration. Stunting of the main root and laterals was a characteristic feature of this toxicity. With the lower concentrations the check was temporary, and the roots eventually made normal growth, as good as that in the control plants. With increasing amounts of palladium chloride the poisoning effect became more persistent, until a concentration was reached which did not allow of any root or shoot recovery.

4. The tolerance of palladium varies with the species, as was indicated by the measure of recovery. Barley appeared to be the least, and

oats the most sensitive of the three cereals tested. Peas responded at much the same concentrations as barley, but broad beans made so complete a recovery from the initial checking that the dry weights were ultimately not reduced even by the strongest concentration tested, the plants being indistinguishable from the controls.

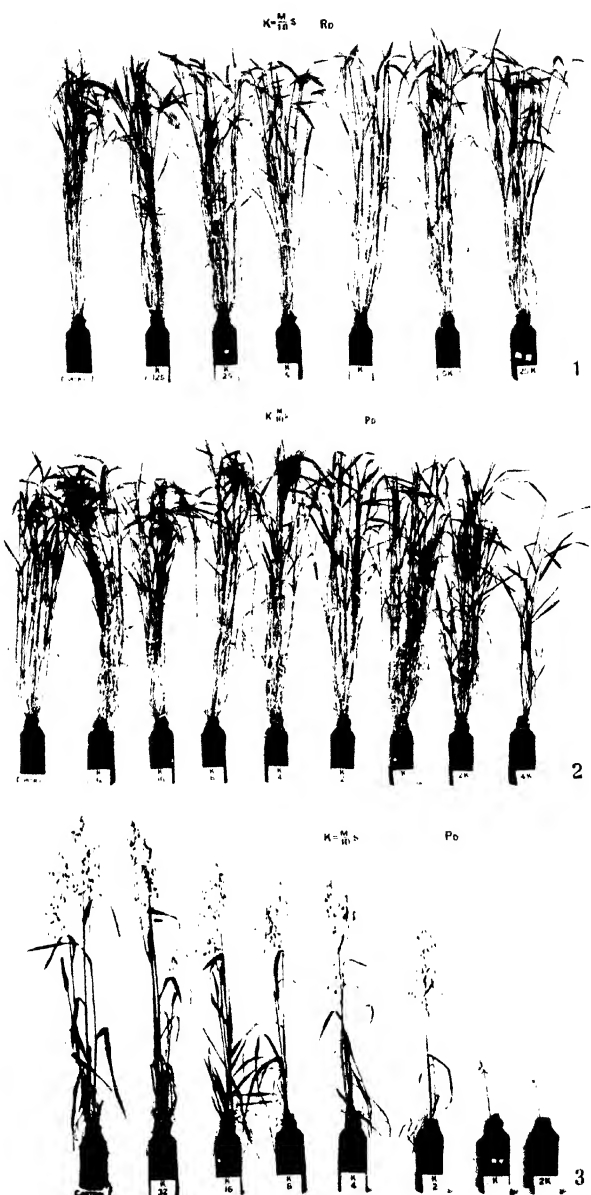
5. The effect of palladium poisoning was similar whether the seeds were germinated in the presence of palladium or whether the seedlings were not introduced to it until they were about a week old.

REFERENCES.

- (1) ALTEN, F. and GOTTWICK, R. (1933). Ein Beitrag zur Frage der Vertretbarkeit des Kaliums durch Rubidium und Caesium für die Pflanzenernährung. *Ernährung der Pflanze*, xxix, Heft 21, pp. 393-9.
- (2) ARENA, M. (1927). Dell' azione di elementi di terre rare sulle piante. *Rend. Accad. Sci. Fis. e Mat. Napoli*, iii, No. 33, pp. 37-9.
- (3) ARNDT, J. (1922). New investigations of the problem of the substitution of potassium in the plant cell by rubidium, as well as the chlorine requirements of buckwheat and barley. *Ernährung der Pflanze*, xviii, 177-80, 191-4. Abs. in *Chem. Zbl.* i, 1923, p. 774.
- (4) BLANCK, E., GIESECKE, F. and HEUKESHOVEN, W. (1933). Vegetationsversuch mit Hafer zur Klärung der Frage nach der Vertretbarkeit des Kaliums durch Rubidium. *Journ. f. Landwirtschaft.* lxxxI, 205-22.
- (5) BOKORNY, T. (1912). Ueber die physiologische Einwirkung einiger Neutralsalze von Alkali und Alkalierdmetallen auf grüne Pflanzen. *Biochem. Z.* xliii, 453-77. Abs. in *Journ. Chem. Soc.* cii, No. 600, ii, p. 975.
- (6) — (1925). Verschiedenes ueber Keimung der Gerste und Ernährung der Hefe. *Allgem. Brauer. u. Hopfenztg.* lxxv, 59-61. Abs. in *Chem. Zbl.* i, 2193; *Chem. Abs.* xx, 2182.
- (7) BRENCHELEY, W. E. (1910). The influence of copper sulphate and manganese sulphate upon the growth of barley. *Ann. Bot.* xxiv, No. xev, pp. 571-83.
- (8) — (1914). On the action of certain compounds of zinc, arsenic, and boron on the growth of plants. *Ibid.* xxviii, No. cx, pp. 283-301.
- (9) — (1917). Organic plant poisons. I. Hydrocyanic acid. *Ibid.* xxxi, Nos. cxxiii and cxxiv, pp. 447-56.
- (10) — (1918). Organic plant poisons. II. Phenols. *Ibid.* xxxii, No. cxxvi, pp. 259-78.
- (11) BRENCHELEY, W. E. and WARINGTON, K. (1927). The rôle of boron in the growth of plants. *Ibid.* xli, No. clxi, pp. 167-87.
- (12) COUPIN, H. (1901). Sur la toxicité des composés de l'argent, du mercure, de l'or, du platine et du palladium à l'égard des végétaux supérieurs. *C.R. Soc. Biol. Paris*, liii, No. 17, pp. 509-10.
- (13) GERBER, C. (1911). The action of the alkali metal salts upon the saccharification of starch by proteolytic ferments. VI. Salts of rubidium, caesium, and lithium. *Ibid.* lxx, No. 18, pp. 826-8. Abs. in *Zbl. Biochem. u. Biophys.* ii, No. 23, p. 945; *Exp. Stat. Rec.* xxvi, 309.
- (14) GRANDEAU, L. (1862). (a) Sur la présence du rubidium dans certaines matières alcalines de la nature et de l'industrie. (b) Sur la présence du rubidium dans un certain nombre de végétaux (betterave, tabac, thé, café, raisins). *C.R. Acad. Sci.* liv, 450-52 and 1057-59.

416 *Effect of Rubidium Sulphate etc. on the Growth of Plants*

- (15) JOHNSTON, E. S. and DORE, W. H. (1928). The relation of boron to the growth of the tomato plant. *Science*, LXVII, 324-5.
- (16) LIPPMANN, E. O. VON (1888). Ueber einige seltenere Bestandtheile der Rübenasche. *Ber. deut. chem. Ges.* xxi, 3492.
- (17) LOEW, O. (1878). Kann das Rubidium die physiologische Function des Kaliums in der Pflanzenzelle übernehmen? *Landw. Versuchs.* xxi, 389-95.
- (18) — (1898). Zur Frage der Vertretbarkeit von Kaliumsalzen durch Rubidiumsalze bei niederen Pilzen. *Bot. Cbl.* LXXIV, No. 7, pp. 202-5.
- (19) — (1903). Ueber die physiologische Wirkung des Chlorrubidiums auf Phanerogamen. *Tokyo Coll. Agric. Bull.* No. 5, pp. 461-5.
- (20) MCHARGUE, J. S. and CALFEE, R. K. (1932). Manganese essential for the growth of *Lemna major*. *Plant Phys.* vii, 697-703.
- (21) MOSER, L. and RITSCHEL, E. (1927). Ueber die quantitative Analyse des Rubidiums und Caesiums. *Z. anal. Chem.* LXX, 184-9. Abs. in *Brit. Chem. Abs.* A, p. 222.
- (22) PFEIFFER, E. (1872). Ueber die Gewinnung von Rubidium aus den Rübenaschen. *Dingl. Polyt. Journ.* CCVI, 498.
- (23) — (1872). Ueber das Vorkommen von Rubidium in den Runkelrüben. *Archiv der Pharmacie*, Zweite Reihe, cl Band.
- (24) RISSE, —. (1865). Quoted in Sachs: *Experimental-Physiologie der Pflanzen*, 153-4.
- (25) RIVIÈRE, G. and BAILHACHE, G. (1913). De l'influence des substances catalytiques. *Journ. Soc. Nat. Hort. France*, 4 Sér., xiv, 728-8.
- (26) ROBINSON, W. O. (1914). The inorganic composition of some important American soils. *U.S. Dept. Agric. Bull.* No. 122, p. 27. Abs. in *Exp. Stat. Rec.* xxxi (1914), 719-20.
- (27) — (1918). A proximate quantitative method for the determination of rubidium and caesium in plant ash. *Journ. Indus. and Engin. Chem.* x, 50, 51. Abs. in *Exp. Stat. Rec.* xxxviii, 412.
- (28) ROBINSON, W. O., STEINKOENIG, L. A. and MILLER, C. F. (1917). The relation of some of the rarer elements on soils and plants. *U.S. Dept. Agric. Bull.* No. 600, p. 25. Abs. in *Exp. Stat. Rec.* xxxviii, 409.
- (29) SAUTON, B. (1912). Influence comparée du potassium, du rubidium et du caesium sur le développement et la sporulation de l'*Aspergillus niger*. *C.R. Acad. Sci.* clv, 1181-3. Abs. in *Exp. Stat. Rec.* xxviii, 527.
- (30) SCHARER, K. and SCHROPP, W. (1933). Sand- und Wasserkulturversuche mit Lithium und Rubidium unter besonderer Berücksichtigung einer etwaigen Ersetzbarkeit des Kaliums durch diese beiden Elemente. *Ernährung der Pflanze*, xxix, Heft 22, pp. 413-36.
- (31) THOMAS, W. (1923). Ultimate analysis of the mineral constituents of a Hagerstown silty clay loam soil and occurrence in plants of some of the elements found. *Soil Sci.* xv, No. 1, pp. 1-18. Abs. in *Exp. Stat. Rec.* xlix, 617.
- (32) WATERMAN, H. J. (1912). Action of hydrogen ions, boric acid, copper, manganese, zinc and rubidium on the metabolism of *Aspergillus niger*. *K. Akad. v. Wetensch. Amsterdam, Proc. Sect. Sci.* xv, pt. 1, pp. 753-64. Abs. in *Exp. Stat. Rec.* xxx, 630.
- (33) WILKE-DÜRFURT, E. (1912). Ueber ein spektralanalytisches Verfahren zur Bestimmung kleiner Mengen Rubidium neben viel Kalium. *Z. Anorg. Chem.* LXXV, 132-40. Abs. in *Journ. Chem. Soc.* cii, ii, p. 686.
- (34) WOLKENHAUER, W. (1924). Ueber den Einfluss von Reizstoffen auf das Längenwachstum der Wurzeln. *Bot. Arch.* vi, 233-74. Abs. in *Bot. Abst.* xiv, 901; *Chem. Abs.* xx, 1647.



BRENCHLEY.—THE EFFECT OF RUBIDIUM SULPHATE AND PALLADIUM CHLORIDE
ON THE GROWTH OF PLANTS (pp. 398-417).

EXPLANATION OF PLATE XX.

- Fig. 1. Effect of increasing concentrations (left to right) of rubidium sulphate on the growth of barley. (See Table I.)
- Fig. 2. Effect of increasing concentrations (left to right) of palladium chloride on the growth of barley. (See Table III.)
- Fig. 3. Effect of increasing concentrations (left to right) of palladium chloride on the growth of oats. (Concentrations as for barley, Table III.)

(Received April 24th, 1934.)

Studies in the Absorption of Calcium from Nutrient Solutions with Special Reference to the Presence or Absence of Boron.

BY

KATHERINE WARINGTON, M.Sc.

(*Botanical Department, Rothamsted Experimental Station.*)

With eight Figures in the Text.

CONTENTS.

	PAGE
I. INTRODUCTION	744
A. GENERAL OUTLINE OF THE EXPERIMENTS	744
B. ANALYTICAL PROCEDURE	746
II. EXPERIMENTAL DATA	747
A. CALCIUM ABSORBED AT DIFFERENT STAGES OF GROWTH FROM A COMPLETE NUTRIENT SOLUTION	747
(a) Boron supplied	747
(1) Actual uptake of calcium	747
(2) Uptake of calcium per cent. dry matter	751
(b) No boron	752
(1) Actual uptake of calcium	752
(2) Uptake of calcium per cent. dry matter	753
B. CALCIUM ABSORBED FROM A SOLUTION OF CALCIUM CHLORIDE ONLY	754
(a) Boron supplied	754
(b) No boron	754
C. EFFECT OF THE FREQUENCY OF RENEWAL OF THE NUTRIENT SOLUTION ON THE QUANTITY OF CALCIUM ABSORBED	755
(a) Boron supplied	755
(b) No boron	757
D. EFFECT OF THE CONCENTRATION OF CALCIUM SUPPLIED ON THE QUANTITY OF CALCIUM ABSORBED	758
(a) Boron supplied	758
(b) No boron	761
E. CONFIRMATORY DATA OBTAINED WITH THE USE OF SPECTROGRAPHIC METHODS OF ANALYSIS	763
F. COMPARISON BETWEEN THE NITROGEN, POTASSIUM, AND CALCIUM CONTENT AT DIFFERENT STAGES OF GROWTH	763
(a) Nitrogen	764
(1) Boron supplied	765
(2) No boron	767

(b) Potassium	769
(1) Boron supplied	770
(2) No boron	771
III. DISCUSSION	772
IV. SUMMARY	774
LITERATURE CITED	775

I. INTRODUCTION.

SOME investigations into the role of boron in plant growth which were the subject of a previous paper (1), gave rise to the suggestion that a definite association existed between this element and the absorption or utilization of calcium. This conclusion seemed warranted as, judging from the dry weight produced, *Vicia faba* plants appeared able to make better use of deficient quantities of calcium if boron were also supplied. In contrast to what obtained when other nutrient elements were lacking, death from a shortage of calcium followed more rapidly in the absence than in the presence of boron, and further, symptoms of a deficiency of calcium showed a close resemblance to those of a lack of boron in that both affected the apical portions of the shoot first, and caused a somewhat similar discoloration and withering. Both Reed and Haas (31) and Nightingale et al. (25), working with young orange trees and tomatoes respectively, also describe apical death as characteristic of a shortage of calcium, while McMurtrey (22) shows how the effects of a deficiency of calcium and boron may be distinguished in the case of tobacco. In the case of a deficiency of any of the other essential elements the appropriate symptoms either made their first appearance in the lower parts of the plant or were in other ways readily distinguished from those due to a deficiency of boron. No actual analyses were carried out in these experiments, and it seemed desirable to make a further study of this possible calcium-boron association. Quantitative estimations were therefore carried out to determine the amount of calcium absorbed by the plant at different stages of growth and under varying nutritional conditions, both in the presence and absence of boron. Water-culture methods were used throughout, as by this means the quantity of calcium supplied could be most readily controlled and the absorption most accurately measured.

The hope of obtaining a clearly defined answer to the problem was, however, not realized, but results of considerable interest were secured which are presented in the following paper.

A. GENERAL OUTLINE OF THE EXPERIMENTS.

A modification of the usual Rothamsted solution¹ was used, calcium

¹ KNO₃ 1.0 grm. KH₂PO₄ 0.3 grm. K₂HPO₄ 0.27 grm. MgSO₄·7H₂O 0.5 grm. CaCl₂ as required. FeCl₃ 0.04 grm. Distilled water 1,000 c.c.

chloride being substituted for the sulphate so as to obtain more accurate measurement of the calcium supplied, and sodium chloride being omitted in order to avoid excess chlorine ions. This solution was made up in bulk *without* the addition of the calcium or ferric salts and the culture bottles (capacity 600 c.c.) filled. Calcium chloride, of which a standardized stock had been prepared, was then added with a pipette as desired, followed by a uniform quantity of ferric chloride solution to each bottle. The pH value of the solution was 6.2, and no alteration in this figure was obtained when the concentration of calcium was reduced. Single plants only were set up in each bottle and excellent growth was made considering the comparatively late season of the year (September–October or November) at which the experiments were carried out. No artificial light was employed.

Since *V. faba* had been chiefly grown in the earlier work, the same plant and variety (Sutton's Prolific Longpod) was used in the present experiments. The seed was graded by weight, but as such a large number of plants was required a somewhat wide range was unavoidable. Care, however, was taken to ensure random distribution of the seeds throughout the different treatments.

In the first season (1930) the nutrient solution supplied contained 52.49 mg. calcium per plant, and special attention was paid to the amount of this element absorbed at different stages of growth when the nutrient medium was both unchanged or renewed at regular intervals. A parallel study of absorption from a pure solution of calcium chloride was carried out at the same time. Each of the treatments was repeated with the addition of 1 p.p.m. boric acid, five or ten plants serving as the unit. The treatments may be summarized as follows:

	Treatment.	Duration.
(i) Nutrient solution.	Renewed weekly after first 2 weeks ¹	9 weeks
(ii) " "	" " " 3 "	9 "
(iii) " "	Renewed fortnightly after first 3 weeks	9 "
(iv) " "	Unrenewed	2-5 "
(v) Calcium chloride solution alone	Unrenewed	4 "

In the following year (1931) the influence of the quantity of calcium supplied on the amount of calcium absorbed by the plant was the chief question studied. The same basal culture solution was used as in the previous season, but the calcium chloride content was varied so as to supply one of the following amounts of calcium:

54.365	mg. calcium per plant (afterwards referred to as full quantity)
27.1825	" " " " $\frac{1}{2}$ "
13.5913	" " " " $\frac{4}{8}$ "
6.7957	" " " " $\frac{1}{8}$ "

¹ Experience had shown that three weeks was a most satisfactory interval to allow before giving the first renewal of solution, but this additional series was included in case appreciable absorption of calcium had occurred during the first fortnight.

As before, the whole series was repeated with the addition of 1 p.p.m. boric acid. The plants were carried on for five weeks only, as, except for the occasional addition of a trace of ferric chloride, no renewal of the solutions was made.

Growth was rather less vigorous than in the previous year, as the dry-weight figures show (Table II), but the plants were entirely healthy and had nearly reached the flowering stage at the end of the five weeks' growth. Although direct comparison between the performance of the plants in the two seasons cannot be made owing to the inevitable differences in external conditions (which would necessarily affect the rates of growth and absorption) and also to the slightly larger amount of calcium supplied as the 'full' quantity in the second season, yet the results from the two-years' investigations, where repeated, are consistent (Table II), so that the additional support is afforded to the accuracy of those other parts of the experiment for which no repetition was possible.

B. ANALYTICAL PROCEDURE.

The used solutions were reserved for analysis every time that plants were harvested or were transferred to a fresh bottle of solution for further growth. As it was of equal importance that no calcium, whether as solution or precipitate, should be left adhering to the plants in either case, the roots were rinsed with a 2 per cent. solution of HCl, followed by distilled water and the washings added to the appropriate solution before analysis. No apparent damage to later growth resulted from this treatment. Two cubic centimetres of concentrated HCl were then added to each bottle preparatory to analysis, in order to ensure the complete solution of all the calcium present, and to prevent the growth of fungi and algae during the interval before the estimations were made.

As most of the concentrations were too weak to ensure sufficient calcium being left in a single bottle for accurate estimation, the solutions from two, three, or five plants were bulked before analysis in the case of the one-half, one-quarter, and one-eighth doses of calcium respectively, triplicate determinations being carried out. Separate estimations, however, were made on the solutions from plants receiving the full supply of calcium, five replicates being available in this case.

After evaporation to a convenient volume the calcium was precipitated as oxalate and estimated by titration with standard potassium permanganate after Chapman's method (4). With the exception of the solution containing the largest concentration of calcium chloride, the high proportion of magnesium to calcium offered some difficulty, as magnesium oxalate tended to be precipitated at the same time as the calcium salt. This trouble was satisfactorily overcome by dissolving the mixture of

magnesium and calcium oxalates in hot HCl and repeating the precipitation as before, the calcium oxalate then being obtained pure.

As a further check on the methods employed, estimations were carried out on the plant ash to see if the loss of calcium from the nutrient medium could be accounted for by that found in the plant itself. The results are given in Table I. Allowance for the initial calcium content of the seed must, of course, be made before comparing the two sets of calcium values. The variation between the dry weights of individual seeds was large, so that the figure for the calcium content per seed is necessarily a mean value only, but taking this into consideration the agreement would seem to be satisfactory.

TABLE I.

Comparison between the Calcium in the Plant Ash and that Lost from the Nutrient Solution after Four Weeks' Growth.

	(mg. per plant. Average of 5.)		Ca Uptake. ¹	
	Seed.	Whole plant.	As found in plant less seed.	As lost from solution.
+ B	2.0	16.7	14.7	15.1
No B	2.0	9.6	7.6	7.1

II. EXPERIMENTAL DATA.

A. CALCIUM ABSORBED AT DIFFERENT STAGES OF GROWTH FROM A COMPLETE NUTRIENT SOLUTION.

(a) *Boron supplied.*

(1) Actual uptake of calcium.

In the case of plants whose culture solution was renewed regularly every week from the end of the second week, and which, therefore, always had access to an ample supply of nutrients, the calcium absorbed increased (with a single exception) up to the seventh week, remained approximately at the same level for a further seven days and then fell significantly during the last, i.e. the ninth week (Fig. 1). By this time the plants were well grown and in flower, but light conditions were becoming unsuitable for vigorous growth, so the experiment was discontinued and data for more mature plants are not available. Burd (3), working with barley, records a similar decrease in the rate of absorption of nutrients after the ninth week of growth, actual loss occurring in the case of calcium. Confirmation of the seventh week as the period of maximum calcium absorption under

¹ In the case of the solutions the average is the mean of 5 separate determinations, but the values for the ash analyses are the means of duplicate determinations on material bulked from 5 plants.

these particular experimental conditions was provided by five other plants grown simultaneously, whose treatment was identical except for the fact that the weekly renewal of their solution was not begun until the third

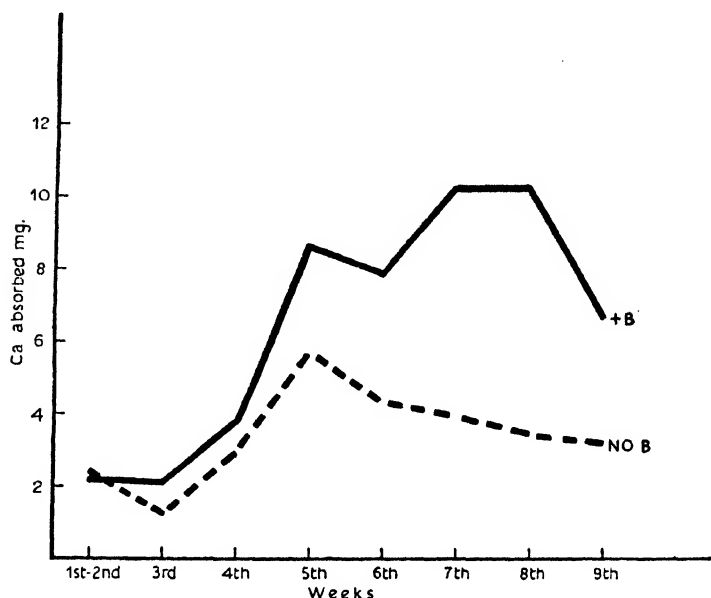


FIG. 1. Calcium absorbed by *Vicia faba* in successive weeks from a complete nutrient solution renewed weekly. 1930.

instead of the second week. A slight check in the uptake of calcium occurred in both sets of plants during the sixth week, and although a statistical examination of the figures showed that any actual decrease lies within experimental error, yet it is evident that at this period the rate of increase in the absorption of calcium was less vigorous than during either the preceding or following week. Calcium uptake would, therefore, seem to be periodic in nature, as has been generally found by a number of workers with other elements and plants (see Lundegårdh (21)). The failure of Redfern (30) to obtain evidence for periodicity in uptake is probably to be accounted for by the short period (36–84 hours) for which her experiments were continued. Fonder's work (8) perhaps offers the closest comparison with the present results, as he also used the bean plant. With a variety of soil types he found a reduction in percentage of calcium in the shoot at budding or fruiting time, which was followed by a rise when complete maturity was attained. It is, therefore, possible that in the present case this final rise was missed owing to the plants being harvested when flowering was reached.

Where the culture solution was renewed every fortnight, after remaining unchanged for the first three weeks, the general course of the curve closely resembled that where fresh solution was supplied weekly, viz. the maximum

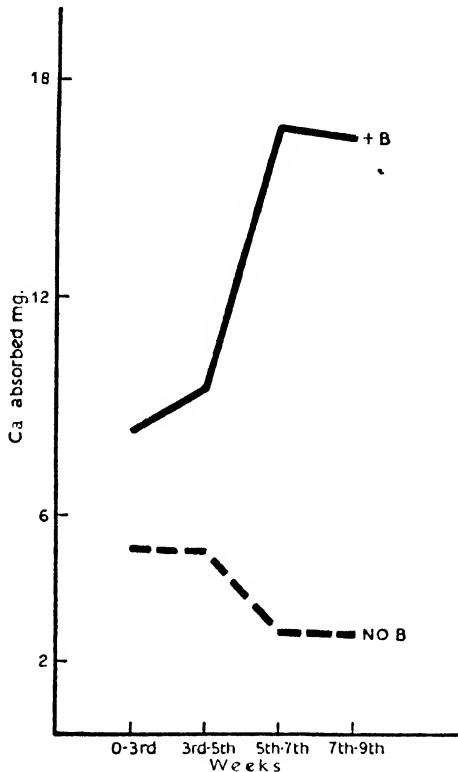


FIG. 2. Calcium absorbed by *V. faba* during successive growth periods from a complete nutrient solution renewed fortnightly. 1930.

uptake of calcium was reached by the seventh week, and although no definite decrease took place during the following two-week period, at least no further rise occurred (Fig. 2).

In the case of plants whose solutions were not renewed at all, figures are available for the first five weeks only, as the general nutrient deficiency which sets in about this date would have inevitably vitiated the results if the plants had been grown on longer. In the 1930 unrenewed series a steady increase in the quantity of calcium absorbed took place with each additional week for which the plants were grown (Fig. 3), and, further, the amount taken up per week also increased up to the end of the experiment (Table II). When this series was repeated in the following year, a drop in the uptake of calcium occurred during the fifth week, so that it seems

probable that the figure for the final week in the previous season had been a maximum point. In this connexion it is interesting to note that the actual amount of calcium absorbed during this probable peak period

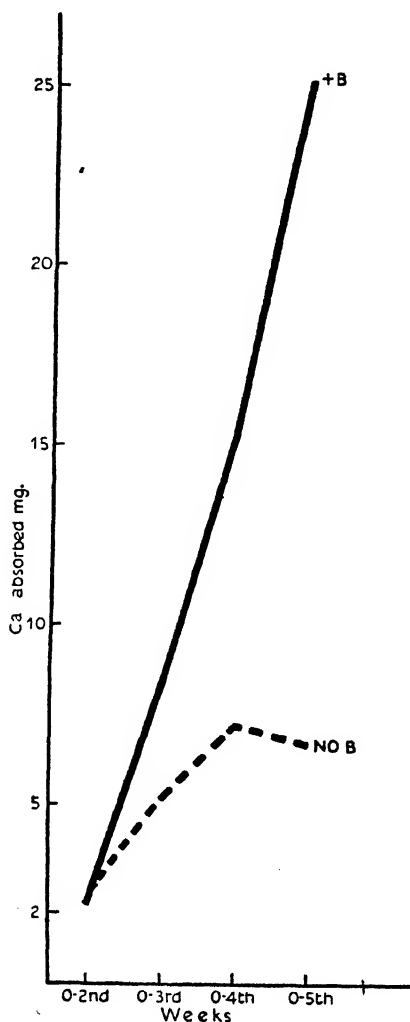


FIG. 3. Calcium absorbed by *V. faba* during progressively increasing periods of growth from a complete nutrient solution not renewed. 1930.

(10 mg. per plant) was practically identical with that taken up by plants receiving weekly renewed solutions during *their* week of maximum absorption (Fig. 1), although in the latter instance the highest point was not reached until a fortnight later.

Many factors can account for the more rapid attainment of the peak period of absorption by the plants in unrenewed solutions, the most important of which is probably the absence of the disturbing factor of the actual renewal itself, especially in the early stages of growth, as will be seen in a later section. The greater reduction in the concentration of all nutrients, the increased variation in balance and pH value of the unchanged medium compared with that which was frequently renewed, would also account for differences in the quantity of calcium absorbed, and the falling off in calcium uptake in the fifth week of the unrenewed series in 1931 is probably to be attributed to these or seasonal factors rather than to any shortage of calcium itself. In support of this, only 25.3 per cent. of the total calcium supplied had been taken up by the end of the fourth week when the absorption began to decrease, although it is of course possible that such factors as are referred to above, increase in alkalinity, changes in balance, concentration, &c., might have induced a deficiency of *available* calcium as distinct from total calcium.

(2) Uptake of calcium per cent. dry matter.

In order to eliminate the effect of individual variation in size and thereby to afford a stricter comparison between the performance of plants receiving different treatments, the calcium absorbed has, where possible, been reckoned as a percentage of the dry matter produced (Table II). Calculated on this basis, the uptake of calcium in the unrenewed series followed the same course as the actual calcium absorbed, i.e. increased with every additional week of the five-week period for which the plants were grown in the 1930 series, no further rise occurring after the fourth week in the repetition experiment in the succeeding year.

TABLE II.

Calcium Absorbed from a Complete Nutrient Solution (with Boron) and Dry Weight Produced at Different Stages of Growth.

Average of 5 plants.			(Solution not renewed.)	
Age weeks.	Actual Ca absorbed.		Dry weight.	Ca absorbed per cent. dry weight.
	Total.	Weekly increment. mg.		
1930 {	2	2.2	1.43	0.154
	3	8.3	2.09	0.397
	4	15.1	3.15	0.479
	5	25.1	4.35	0.577
1931 {	2	2.2	1.15	0.188
	3	6.2	1.57	0.395
	4	13.8	2.42	0.570
	5	17.1	2.39	0.504
3 D				

Figures for more mature plants are afforded by the series whose solutions were renewed fortnightly or weekly. In the former case, the calcium absorbed per cent. of dry weight produced during the sixth to ninth weeks showed a considerable advance on that taken up by the same plants during the previous five-week period (Table III). The results from the weekly renewed series (Table IV), on the other hand, are at first sight contradictory, as the calcium absorbed per cent. of dry weight after nine weeks is very little, if at all, larger than that absorbed in the first five weeks by plants in either unrenewed or fortnightly renewed solutions (Tables II and III). Closer inspection, however, reveals the fact that the *average* value for the complete nine weeks in the fortnightly renewed series is practically identical with that in the weekly renewed set during the same period, showing that the fluctuation in uptake had been lost sight of when the final value only was available. Intermediate figures, however, were unable to be obtained as it was impossible to determine dry weights at successive stages when identical plants were under observation for a series of weeks.

TABLE III.

Calcium Absorbed from a Complete Nutrient Solution and Dry Matter Produced at Different Stages of Growth.

Average of 5 plants.		(Solutions renewed fortnightly.)			Average.
		Total Ca absorbed. mg.	Dry weight. gram.	Ca absorbed per cent. dry weight.	
+ B	{ 1st-5th week	17.8	4.36	0.408	} 0.584
	{ 6th-9th week	33.1	4.36	0.759	
No B	{ 1st-5th week	10.1	2.80	0.396	} 0.379
	{ 6th-9th week	5.5	1.39	0.396	

TABLE IV.

Calcium Absorbed from a Complete Nutrient Solution and Dry Matter Produced after Nine Weeks' Growth.

Average of 10 plants.		(Solutions renewed weekly.)		
		Total Ca absorbed. mg.	Dry weight. gram.	Ca absorbed per cent. dry weight.
+ B		54.83	9.22	0.588
No B		26.65	4.84	0.548

(b) *No boron.*

(1) Actual uptake of calcium.

A lack of boron brought about distinct differences in the uptake of calcium. In the first place the amount of calcium absorbed increased with

age up to the fourth or fifth week only, irrespective of the frequency with which the culture solution was renewed, and further, the actual quantity absorbed each week was smaller and the rate of increase in the uptake slower than in the case of plants supplied with boron (Figs. 1, 2, and 3 and Table V). Where it was possible to grow the cultures on for nine weeks (weekly and fortnightly renewed series) the quantity of calcium taken up steadily decreased after the fourth or fifth week until the end of the experiment.

TABLE V.

Calcium Absorbed from a Complete Nutrient Solution (without Boron) and Dry Weight Produced at Different Stages of Growth.

Average of 5 plants.		(Solution not renewed.)			
Age weeks.		Actual Ca absorbed.		Dry weight.	Ca absorbed per cent. dry weight.
		Total.	Weekly increment.		
		mg.			gm.
1930	2	2.6	2.6	1.45	0.179
	3	5.1	2.5	1.74	0.293
	4	7.1	2.0	2.30	0.297
	5	6.6	-1.5	2.42	0.273
1931	2	2.2	2.2	1.19	0.193
	3	3.5	1.3	1.33	0.263
	4	5.5	2.0	1.76	0.313
	5	6.0	0.5	2.02	0.297

The time at which any significant difference appeared between the calcium absorbed by plants grown with or without boron depended, as will be seen in a later section, on the frequency with which the culture solution was renewed.

(2) Uptake of calcium per cent. dry matter.

If the calcium taken up is reckoned as a percentage of the dry matter, a stricter comparison is possible with the results produced from plants grown with boron already described. On this basis the absorption from unrenewed solutions without boron is seen to increase until the third or fourth week only, i.e. as far as the 1930 experiment was concerned the maximum tended to be reached somewhat earlier and was significantly lower than where boron was supplied, or, in other words, a lack of boron affected the uptake of calcium more seriously than the production of dry matter (cf. Tables II and V).

As regards older plants, Table III shows that with solutions renewed fortnightly the calcium absorbed per cent. of dry matter produced was approximately the same during the sixth to ninth week, as it had been during the previous five-week period, in contrast to that which obtained with the boron-treated set. A lack of boron, therefore, had its greatest influence

during the latter part of the growth period when it depressed the uptake of calcium more than the production of dry weight. The final value after nine weeks' growth, for the plants with solutions renewed weekly, appears entirely anomalous (Table IV), as it was considerably larger than that obtained in the first five-week period for the other plants grown without boron (Tables III and V), and in consequence is apparently contradictory to the findings of Table IV, where no increase was obtained after the fifth week of growth. The explanation probably lies in the fact that the rate of renewal of the solution has an important bearing on the quantity of calcium absorbed, particularly in the case of plants grown without boron.

B. CALCIUM ABSORBED FROM A SOLUTION OF CALCIUM CHLORIDE ONLY.

In order to study the uptake of calcium in the simplest manner possible, and previous experience having shown that healthy growth could be maintained for a limited period under such conditions, ten plants were set up in a pure solution of calcium chloride, each receiving the same amount of calcium per plant (52.49 mg.) as the series in the complete nutrient medium had done. As before, one half of the plants were given 1 p.p.m. boric acid in addition.

(a) *Boron supplied.*

The quantity of calcium absorbed was considerable, the amount taken up after four weeks closely resembling that removed by the more actively growing plants in complete nutrients during a three-week period. The dry weight laid down by the latter in this time, however, was distinctly larger, so that the relative absorbing capacity of the plants in the pure solution was the greater (Table VI).

(b) *No boron.*

The rapidity with which the symptoms of boron deficiency appeared was striking, the inhibitory effect on root growth being particularly well marked. The earliness with which dying set in was reflected in the very small quantity of calcium absorbed, the difference between the uptake of plants grown with and without boron being much more marked in the set in the pure calcium chloride solution than in those plants grown in the complete nutrient medium. An illustration of this is given in Table VI. If, for example, the stage at which the plants with boron had each absorbed approximately 8 mg. calcium is taken as a standard, it will be seen that during the same interval the set in the pure solution, without boron, had taken up only 2.39 mg., whereas given full nutrients the corresponding uptake in the absence of boron was 5.11 mg., or rather more than twice the

same amount absorbed from the single salt solution. Indeed, comparison has to be made between plants with one week difference in age in the complete nutrients, in order to get as marked a distinction between those grown with and without boron. The fact that the absorption of 8 mg. was effected during a slightly different period under the two different nutrient conditions does not alter the question. The calcium absorbed from the pure solution per cent. of dry weight laid down was also much reduced in the absence of boron, so that a lack of this element was clearly exerting a greater inhibitory influence on calcium absorption than on dry matter production. The reduction in the root surface, however, may partly account for this, as the short, thick roots characteristic of the plants grown without boron would possess a smaller surface-absorbing area than the finely divided root system of the normal plant, and dry-weight figures would not give any indication of such differences.

TABLE VI.

Comparison between the Uptake of Calcium from a Solution of Calcium Chloride and a Complete Nutrient Medium, the Calcium Supplied being Identical.

	Age weeks.	Total Ca absorbed. mg.	Dry weight. gm.	Uptake Ca per cent. dry weight.	
+ B	4	8.48	1.37	0.61	Calcium chloride solution only.
No B	4	2.39	1.17	0.20	
+ B	3	8.26	2.09	0.39	Complete nutrients.
No B	3	5.11	1.74	0.29	
No B	2	2.87	1.45	0.20	

The calcium absorbed per cent. of dry weight produced was, therefore, reduced by the presence of other nutrients where boron was supplied. At the same time the appearance of the deficiency symptoms was delayed if it were withheld, so that some association between boron and calcium is suggested.

C. EFFECT OF THE FREQUENCY OF RENEWAL OF THE NUTRIENT SOLUTION ON THE QUANTITY OF CALCIUM ABSORBED.

(a) *Boron supplied.*

As is evident from Table VII, the total amount of calcium absorbed over a nine-week period in the presence of boron was practically identical whether the solution was renewed at weekly or fortnightly intervals.

TABLE VII.

Effect of Frequency of Renewal of Nutrient Solution on the Absorption of Calcium over a Nine-week Period.

Average of 5 plants. Period (weeks).	With B.		No B.	
	Ca absorbed (mg. per plant).			
	Renewal.	Renewal.	Renewal.	Renewal.
	Weekly. ¹	Fortnightly. ²	Weekly. ¹	Fortnightly. ²
0-3	4.3	8.3	3.7	5.1
3-5	12.4	9.5	8.7	5.0
5-7	17.9	16.7	8.2	2.8
7-9	16.7	16.4	5.7	2.7
Total	51.3	50.9	26.3	15.6
Final dry weight per plant (gram.)	8.82	8.72	5.12	4.19
Ca absorbed per cent. dry weight	0.582	0.584	0.513	0.373
Per cent. absorption of Ca supplied	12.2	24.2	6.3	7.4

The final dry weights of the two sets of plants were also identical, so that the calcium absorbed per cent. of dry matter produced was similar in the two cases. Only in the percentage of the calcium supplied that was taken up by the plants was any significant difference in the two treatments noticeable. A more detailed investigation, however, shows that a change of solution at an early stage, such as the second week, resulted in a temporary check in calcium absorption, for the plants which remained in their original medium for the first three weeks took up approximately double the quantity of calcium in that period compared with those which were given a fresh supply at the end of the second week. It was thought possible that this check might have been partly due to the fact that such young roots were rinsed in a 2 per cent. solution of HCl before being transferred to the fresh supply of solution, but as the same effect occurred to an even more marked degree when comparison was made between older plants and those grown entirely in unrenewed solution, the change itself was probably the important factor.

The unrenewed series, on the other hand, showed a much greater calcium absorption during the first five weeks of growth than either the weekly or fortnightly series (Table VIII). Since the dry weight laid down per plant during this period was identical in the fortnightly and unrenewed sets (the figure for the weekly changed series is not available), it is evident that the plants receiving the less frequent supply of nutrients were the more active in calcium absorption.

Five weeks' growth may seem a somewhat short interval from which to deduce such results, but as the flowering stage had already been reached, a phase of rapid growth is included in this period.

¹ After the first two weeks.

² After the first three weeks.

TABLE VIII.

Effect of Frequency of Renewal of Nutrient Solution on the Absorption of Calcium over a Five-week Period.

	Solution renewed.	No. of renewals.	Total Ca absorbed per plant. mg.	Dry weight. gram.	Ca absorbed per cent. dry weight.	Percent. of supplied Ca absorbed.
+ B	Weekly	4	16.7	not determined	not determined	8.0
	Fortnightly	2	17.8	4.36	0.408	17.0
	Not renewed	1	25.1	4.35	0.577	47.8
No B	Weekly	4	12.4	not determined	not determined	5.9
	Fortnightly	2	10.1	2.80	0.361	9.6
	Not renewed	1	6.6	2.42	0.273	12.6

(b) *No boron.*

In the absence of boron entirely different results were obtained, and before attempting any explanation for this, some reference to the general behaviour of the broad bean plant grown under boron-deficiency conditions must be made. Owing to the considerable quantity of this element stored in the seed, as much as three weeks may elapse before any symptoms of a deficiency appear to the eye. The cotyledons were not removed from the plant in these experiments as the check to growth is so severe. Further, the time of the appearance of the deficiency symptoms and the rate of their progress depends on external conditions, of which the frequency of the renewal of the solutions and light (which has been the subject of a previous paper (35)) are the most important factors. The former only will be dealt with in the present instance. It has often been observed that if the nutrient solution is renewed frequently, a delay in the development of boron-deficiency symptoms generally occurs, the extreme cases being where plants are grown in 'drip' cultures (1) or in unchanged solutions. The reason for this is not quite understood unless it implies the unconscious introduction of a source of boron at each renewal of solution, but for the moment the facts will have to be accepted and allowance made for them in interpreting the results under consideration.

None of the plants grown without boron absorbed as much calcium as those supplied with it, and a further contrast in behaviour was found in that the total quantity of calcium absorbed during the nine weeks of growth was considerably lower in the series where solutions were renewed fortnightly, than in that where fresh nutrients were supplied every week, the reduction occurring chiefly during the latter part of this period (Table VII).

The amount of dry weight laid down during this time, however, was only slightly smaller in the fortnightly than in the weekly renewed series,

with the result that the uptake of calcium per cent. of dry matter was significantly greater in the plants which received a fresh supply of solution every week. Explanation for this is afforded by the behaviour of the plants grown without boron just described, for those dying the more rapidly from a lack of boron (i.e. the fortnightly renewed set) would be expected to show a greater reduction in calcium absorption than in dry matter production as the former process is the more seriously affected by a lack of boron, and further, to show any such differences from the weekly renewed series most clearly when a lack of boron had become really acute, i.e. in the later stages of growth.

A check in calcium uptake was induced by a renewal of the solution at the end of the second week of growth, similar to that described for plants supplied with boron, but as at this early stage no deficiency symptoms had yet appeared, it is not surprising that the behaviour of the two sets of plants was identical.

In the case of the unrenewed series data are available for the first five weeks only. Here definitely less calcium was absorbed than where the cultures were renewed, in contrast to the increase that obtained in the corresponding set of plants grown with boron (Table VIII). It is, therefore, evident that even during the first five weeks of growth, the presence of boron is an important factor, and that an increased rate in the development of deficiency symptoms in its absence is definitely correlated with a reduction in the frequency of the supply of nutrients, and is also reflected in an early falling off in the amount of calcium absorbed by the plant.

In a similar manner, the uptake of calcium per cent. dry weight produced was affected by the frequency of the renewal of the solution and by the presence or absence of boron, being reduced where boron was not supplied and still further reduced if the solutions were not renewed and the effect of a lack of boron thereby enhanced.

D. EFFECT OF THE CONCENTRATION OF CALCIUM SUPPLIED ON THE QUANTITY OF CALCIUM ABSORBED.

(a) *Boron supplied.*

This aspect of the problem was studied in unrenewed solutions only, and the cultures were in consequence not carried on for longer than five weeks. The quantities of calcium tested were 54.365 mg. calcium per plant (given as calcium chloride), and one-half, one-fourth, and one-eighth of this amount, a range which previous experience with the Rothamsted nutrient solution had shown included both ample and deficient supplies of this element.

Analyses were made weekly, beginning at the end of the second week, as the quantity of calcium absorbed before that time was so small as to lie

within the experimental error. From the second to the fifth week both the actual uptake of calcium (Fig. 4) and the uptake per cent. of dry matter were found to be almost directly proportional to the quantity of

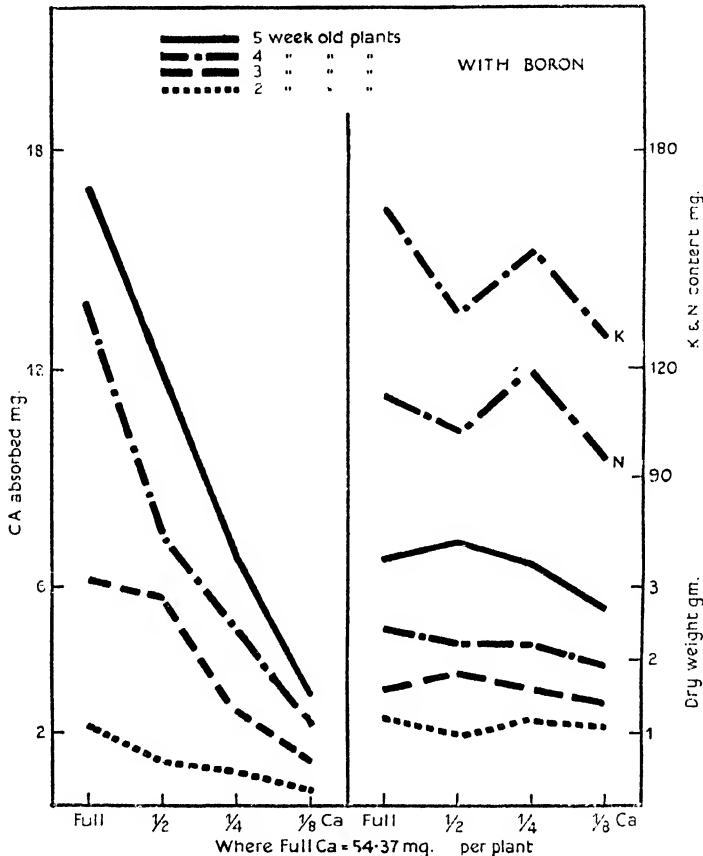


FIG. 4. Relation between calcium absorption, potassium and nitrogen content, dry matter production and the quantity of calcium supplied to *V. faba* for plants of different ages. Solutions not renewed. 1931.

calcium supplied, the course of the curves approximating to a straight line in each case. Many other workers, such as Ginsburg and Shive (11), Newton (24), and Philipson (28), have also found this direct relationship to hold with plants grown in solution cultures, while Fonder (8), Holtz (14), Shedd (34), and others report the same experience with plants in soil. Waynick's (36) and Gile and Ageton's (10) work, however, with solution and soil cultures respectively, shows that this direct relationship does not universally obtain.

The actual absorption also progressed in proportion to the age of the

plant (Fig. 5), which confirms the findings of the previous season and extends the results to smaller concentrations of calcium. By calculation it is evident that with the exception of the half dose, the period of maximum

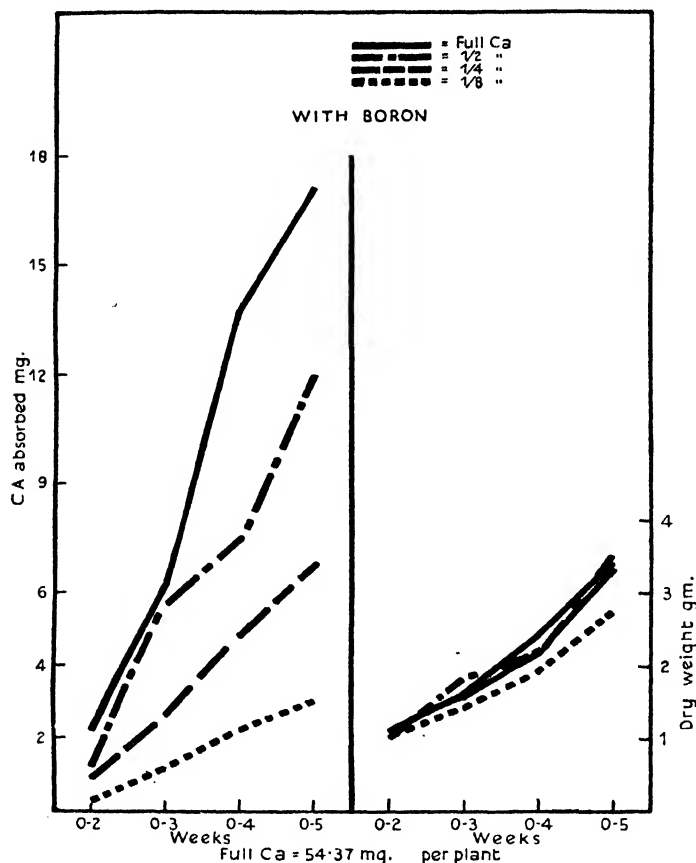


FIG. 5. Relation between calcium absorption, dry matter production, and the age of *V. faba* when supplied with different amounts of calcium. Solution not renewed. 1931.

absorption tended to lie in the fourth week, though no marked maxima occurred except where full calcium was given (Table IX). The reason for the exceptional behaviour in the plants receiving the half dose of calcium is not apparent.

Little variation was shown in the percentage of the calcium provided that was taken up by the plant in each case, except where the highest quantity of calcium was supplied. Here the percentage utilized was lower than in all other cases, which suggests that the calcium provided was in excess of that required (Table X). This view receives support from Day (6) who contends that calcium is commonly given in too high concentration

in nutrient solutions, and from Larson (17) and Hartmann and Powers (13) who found that calcium was most economically utilized when supplied at a concentration of 32 p.p.m., a value not far from that of the half dose of calcium chloride given here.

TABLE IX.
*Progress of Calcium Absorption from Nutrient Solutions Containing
Different Amounts of Calcium.
Solution not Renewed.*

Treatment.		Calcium absorbed per plant per Week. (mg.)				
		1st. & 2nd.	3rd.	4th.	5th.	Total.
With B	Full Ca	2.2	4.0	7.6	3.3	17.1
	$\frac{1}{2}$	1.2	4.5	1.8	4.4	11.9
	$\frac{1}{4}$	0.9	1.7	2.1	2.0	6.7
	$\frac{1}{8}$	0.3	0.9	1.0	0.8	3.0
No B	Full Ca	2.2	1.3	2.0	0.5	6.0
	$\frac{1}{2}$	1.2	2.1	0.3	0.6	4.2
	$\frac{1}{4}$	0.6	1.1	0.7	-0.2 ¹	2.2
	$\frac{1}{8}$	0.2	0.6	0.2	0.0	1.0

TABLE X.
*Relationship between the Quantity of Calcium Supplied and Absorbed
in Five Weeks. 1931.*

Total Ca supplied. mg.	With boron.		No boron.	
	Actual.	Percentage.	Actual.	Percentage.
54.37	17.1	31.5	6.0	11.0
27.18	11.9	43.8	4.2	15.5
13.59	6.7	49.3	2.2	16.2
6.80	3.0	44.1	1.0	14.7

Although the uptake of calcium bore a definite relationship to the quantity of calcium provided even from the second week of growth, the dry weight laid down did not show any such close association, but remained unaffected until the fifth week when the two smaller doses exerted a slightly depressing influence on yield (Fig. 4). It will be seen in a later section that the dry weight showed a closer correlation with the nitrogen than with the calcium content of the plant, which indicates the greater importance of the former element for dry matter production.

(b) *No boron.*

In the absence of boron the quantity of calcium absorbed throughout the five weeks' growth was approximately proportional to that supplied whether the uptake was reckoned as actual calcium (Fig. 6) or as calcium

¹ Negative quantity accounted for since the figure is the difference between the means values of two sets of plants which inevitably showed individual variation.

per cent. of dry matter produced. In this respect the results were similar to those where boron was supplied. The quantities absorbed, however, were very much lower than in the presence of boron except at the first

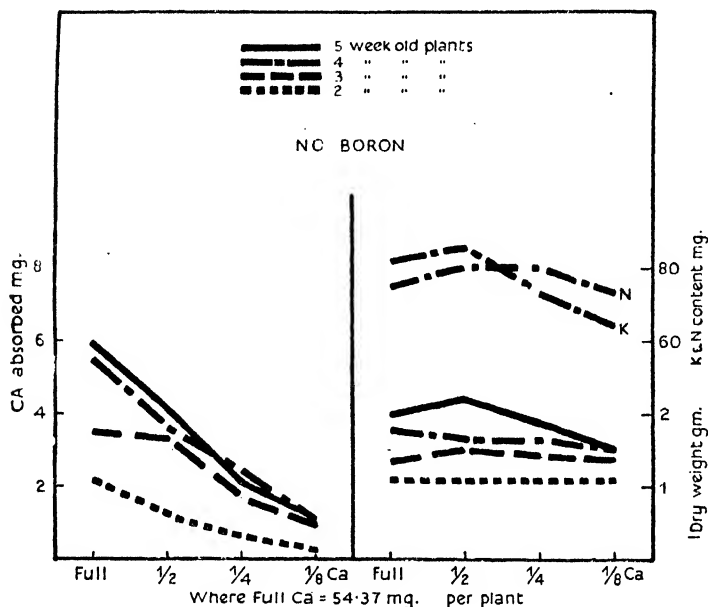


FIG. 6. Relation between calcium absorption, potassium and nitrogen content, dry matter production and the quantity of calcium supplied to *V. faba* for plants of different ages. Solutions not renewed. 1931.

analysis which was made before a deficiency of this element had arisen. The uptake slowly increased with every additional week for which the plants were grown where the two larger doses of calcium were supplied, but reached a maximum at the end of the fourth week, when only the smaller quantity of calcium was given. Except in the case of the highest concentration of calcium, the maximum absorption tended to occur during the third week, that is, a week earlier than where boron was supplied (Table IX). Although the differences between the figures are small, so that deduction from them must be made with caution, yet it is suggestive that dying from a lack of boron began to occur earlier under conditions of deficient calcium, a fact which had already been observed in previous experiments, and to which reference has been made above.

The quantity of calcium presented had little influence on the percentage that the plants absorbed, but the uptake in every case was much reduced by a lack of boron, being approximately only one-third of that which obtained where boron was also supplied (Table X).

E. CONFIRMATORY DATA OBTAINED WITH THE USE OF SPECTROGRAPHIC METHODS OF ANALYSIS.

During the period September to December, 1928, the opportunity arose of studying spectrographic methods of analysis under Professor Lundegårdh at Experimentalfältet, Stockholm; the problem selected for investigation there was similar to that with which the present paper is concerned. Full description of the apparatus and methods have been published (20), (21), so that it suffices to state that the calcium analyses were carried out by means of flame-spectra of solutions in which the plants had been grown, after ensuring that all the calcium present was in the soluble form, i.e. as chloride. *Phaseolus multiflorus* (Blomsterböna tvåfargade) was used as the experimental plant, and the basal culture solution was that employed in the present case, but the quantity of calcium supplied, though varied, was in general rather lower than in the experiments just described. Owing to the comparatively short time available and the late season of the year, no very conclusive results were obtained, although the use of artificial light and heating as the winter set in made it possible to obtain quite vigorous growth. Some brief reference to the results may, however, be of interest, for where they supply confirmatory evidence for those just described above, obtained by chemical methods of analysis, their value is considerably enhanced. In the first place both *Vicia faba* and *P. multiflorus* showed a close agreement in the very large quantity of calcium that they were able to absorb from solutions containing an adequate supply of that element. Whereas, in the presence of boron, *Vicia* plants provided with 52.49 mg. calcium per plant per change of solution absorbed on an average 40.41 mg. during fifty-three days, *Phaseolus*, similarly supplied with 36.58 mg. calcium, took up 35.08 mg. per plant in the same interval. In the absence of boron, the figures were 22.55 and 30.10 mg. per plant for *Vicia* and *Phaseolus* respectively, but as the boron deficiency symptoms developed rather more slowly in the latter species, a greater uptake was to be expected. A directly proportional relation was found between the quantity of calcium supplied and absorbed, which also confirms the results just described. Over a fifty-three-day period in the presence of boron the uptake of calcium per cent. of dry matter produced was 1.96 and 0.220 where the full quantity and one-tenth of it were supplied respectively. The corresponding figures for the plants grown without boron were 1.86 and 0.188, again showing the tendency for the uptake of calcium to be increased in the presence of boron.

F. COMPARISON BETWEEN THE NITROGEN, POTASSIUM, AND CALCIUM CONTENT AT DIFFERENT STAGES OF GROWTH.

In order to avoid the danger of assuming too close an association between the absorption of calcium and the presence of boron in the nutrient

solution, a parallel study was made of the uptake of nitrogen and potassium by the same plants. By this means it was hoped to gain a clearer understanding as to whether the reduction in the calcium taken up by the plants grown without boron was merely the result of a general decrease in absorbing capacity, or whether it indicated a special association between boron and the amount of calcium that the plant could absorb.

(a) *Nitrogen.*

The question of a possible relationship between calcium and nitrogen in nutrition has received attention from several investigators. Gile and Ageton (10) in field experiments found no change in nitrogen content of the crop when the soil was limed, and, similarly, neither Ginsburg and Shive (11) nor Philipson (28), using water-culture methods record any close association between the calcium supplied and the nitrogen absorbed. Newton (24) studied the question from the reverse point of view, but came to similar conclusions, as peas grown with an increased nitrogen supply did not show a correspondingly higher calcium content, although their nitrogen content was raised. Parker and Truog (26), on the other hand, consider that a definite relationship between the two elements does exist and Lipman and Blair (18) found an increased nitrogen content in soy bean where lime had been applied. Further, Nightingale et al. (25) working with the tomato, showed that an absence of calcium from the nutrient medium inhibited the uptake of nitrogen, and also that the nitrogen that was absorbed remained unelaborated as the deficiency of calcium had resulted in a lack of the necessary reductase. Jacobson and Swanback (15) claim that the form in which the nitrogen is presented influences the uptake of calcium, a higher percentage of calcium being found in plants grown with nitrogen in the form of nitrate than where it is given as ammonia.

The principal question in the present experiments, however, was to determine if there were any difference in the relation between the calcium and nitrogen absorbed by plants grown with and without boron.

The nitrogen estimations¹ were made by the Kjeldahl method on the dried plant material, and not determined from analysis of the nutrient solution as in the case of calcium. In order to obtain comparable values of the calcium and nitrogen estimations, one of two courses was open, (a) to subtract the initial nitrogen content of the seed from that found in the plant so as to give the nitrogen in terms of uptake or, (β) to add the initial calcium content of the seed to that absorbed to give the calcium in terms of content. The latter is clearly the more accurate method, as the seed contained so much less calcium than nitrogen (1.8 or 2 mg. calcium as against 60 or 70 mg. nitrogen per seed), and all the figures quoted have been obtained in this manner.

¹ Carried out by the Chemical Department of the Rothamsted Experimental Station.

(1) *Boron supplied.*

A very marked decrease in N/Ca ratio occurred as the plant developed. After nine weeks' growth the ratio had fallen to approximately one-seventh of that originally found in the seed (Table XI), and the values for the successive stages of growth in plants up to the age of five weeks (Table XII), show the same course of events provided the calcium supply was adequate.

TABLE XI.

Actual Calcium and Nitrogen Content of Vicia faba Seed Compared with that of Plants in Flower Grown in a Complete Nutrient Solution Renewed Weekly. 1930.

	Ca.	N.	N/Ca.
	mg per plant.		
Seed	2.0	71.8	35.9
With Boron } after 9 weeks	53.5	293.0	5.48
No Boron }	28.25	173.0	6.12

The figures for the nine- and five-week-old plants (Tables XI and XII respectively) are, however, not strictly comparable, as in the former case the solutions were renewed weekly and the supply of calcium or nitrogen was far in excess of the plants' needs, whereas in the latter instance growth was in unrenowned solutions and the plants were probably subjected to some limitation in nitrogen supply, though no actual chlorosis appeared (Table XIII). The drop in N/Ca ratio in the latter instance, therefore, would tend to be exaggerated, but the closely parallel figures obtained in the succeeding year where no shortage of nitrogen occurred (20 per cent. nitrogen still unabsorbed after five weeks) suggests that the values as given were probably but little affected by any limitation of food supplies.

Where the supply of calcium was extremely deficient, however, the fall in the N/Ca ratio with age was but small, since, although the calcium uptake was at a very much lower level throughout than where the full supply of calcium was given, the nitrogen absorption was only slightly reduced (Table XII). In fact, no correlation was found between the quantity of calcium supplied and the nitrogen content of the plant, as Figs. 4 and 8 show in the case of the four-week-old plants for actual and percentage figures respectively. The nitrogen content, however, corresponded closely with the dry weight production irrespective of the amount of calcium supplied, the correlation, with a single exception, being significant throughout the five weeks' growth. The drop in nitrogen content with the half-dose of calcium is probably partly to be accounted for by individual variation, but it will be noticed that it is also accompanied by a slight fall in dry weight and potash content.

TABLE XII. Calcium, Nitrogen, and Potassium Content of Plants of Different Ages Supplied with Complete and Deficient Calcium. With Boron.

(Solution not renewed.)

Age. Weeks.	Calcium.			Nitrogen.			Potassium.			N/Ca.		K/Ca.	
	Actual (mg.).		Percent, of dry matter.	Actual (mg.).		Percent, of dry matter.	Actual (mg.).		Percent, of dry matter.	Full Ca.	½ Ca.	Full Ca.	½ Ca.
	Full Ca.	½ Ca.		Full Ca.	½ Ca.		Full Ca.	½ Ca.					
1930	Seed												
	2	—	0.125	71.8	—	4.46	—	—	—	35.9	—	—	—
	3	3.8	0.266	85	—	6.02	—	—	—	22.6	—	—	—
	4	10.3	0.494	110.6	—	5.30	—	—	—	10.7	—	—	—
	5	17.1	0.543	150.3	—	4.78	—	—	—	8.8	—	—	—
1931	Seed												
	2	27.1	0.623	146.5	—	3.37	—	—	—	5.4	—	—	—
	3	1.8	0.127	61.1	61.1	4.50	16.95	16.95	1.25	33.9	33.9	9.4	9.4
	4	4.0	0.342	59.8	65.0	5.82	50.74	—	4.41	15.0	31.0	12.7	—
	5	8.0	0.509	83.4	3	5.35	93.63	85.10	5.96	10.4	25.1	11.7	28.4
1931	Seed												
	2	15.6	0.643	113.0	94.5	4.95	164.20	127.90	6.77	7.2	23.6	10.5	32.0
	3	18.9	0.558	126.3	124.6	4.63	189.76	166.90	5.60	6.7	26.0	10.0	34.8

TABLE XIII.

Comparison between the Calcium and Nitrogen Supplied and Absorbed under Different Conditions of Nutrition. 1930.

	Solutions renewed.	Age weeks.	Total Ca per plant.		% Ca used.
			Supplied.	Absorbed.	
			mg.		
With Boron	Weekly	9	419.92	51.3	12.2
	Not renewed	5	52.49	25.1	47.8
No Boron	Weekly	9	419.92	26.3	6.3
	Not renewed	5	52.49	6.6	12.6

	Solutions Renewed.	Age weeks.	Total N per plant.		% N used.
			Supplied.	Absorbed. ¹	
			mg.		
With Boron	Weekly	9	655.6	121.2	18.2
	Not renewed	5	83.2	74.7	89.8
No Boron	Weekly	9	665.6	102.2	15.4
	Not renewed	5	83.2	20.1	24.2

(2) *No boron.*

In the absence of boron a similar fall in the N/Ca ratio occurred as the plant developed from the seed (Table XI), but the decrease was slightly less than where boron was supplied. The tendency for the ratio to be higher is also noticed in the case of the unrenewed series, where a lack of boron exerted a maximum effect (cf. Tables XII and XIV). No question of any shortage of calcium or nitrogen supply arose here, for as will be seen from Table XIII, only a small proportion of the calcium and nitrogen presented had been taken up by the plant. Under conditions where a shortage of calcium obtained, both the nitrogen and calcium contents failed to rise further after the third week of growth, but as in the case of plants supplied with boron, the nitrogen uptake was much less reduced by a shortage of calcium than was the calcium absorption, so that the N/Ca ratio was throughout considerably higher than in the plants grown in the complete nutrient solution (Table XIV). The depressing effect of an absence of boron was, further, more marked on the calcium than on the nitrogen uptake, with the result that N/Ca ratio tended to be higher in the set without than with boron, though this was not so clearly defined as where the calcium supply was adequate. The nitrogen content showed no correlation with the quantity of calcium provided (Figs. 6 and 8), and although growth was so poor in the boron-starved plants that a statistically significant association between the nitrogen content and the dry matter production could not be obtained, yet the results suggested that this held very much in the same way as for the plants supplied with boron.

¹ Figure obtained by subtracting original nitrogen content of seed from nitrogen found in plant.

TABLE XIV. Calcium, Nitrogen, and Potassium Content of Plants of Different Ages Supplied with Complete and Deficient Calcium. No Boron.

(Solution not renewed.)

Age Weeks.	Calcium.		Nitrogen.		Potassium.		N/Ca.		K/Ca.	
	Actual (mg.).	Percent. of dry matter.	Actual (mg.).	Percent. of dry matter.	Actual (mg.).	Percent. of dry matter.	Full Ca.	$\frac{1}{3}$ Ca.	Full Ca.	$\frac{1}{3}$ Ca.
	Full Ca.	$\frac{1}{3}$ Ca.	Full Ca.	$\frac{1}{3}$ Ca.	Full Ca.	$\frac{1}{3}$ Ca.	Full Ca.	$\frac{1}{3}$ Ca.	Full Ca.	$\frac{1}{3}$ Ca.
1930	Seed	—	—	—	—	—	—	—	—	—
	2	4.9	—	—	—	—	—	—	—	—
	3	7.1	—	—	—	—	—	—	—	—
	4	9.1	—	—	—	—	—	—	—	—
	5	8.6	—	—	—	—	—	—	—	—
1931	Seed	1.8	0.127	0.127	61.1	4.50	16.95	1.25	33.9	9.4
	2	4.0	0.352	0.175	68.6	5.78	52.05	—	17.2	31.3
	3	5.3	0.398	0.290	73.5	5.52	60.65	62.4	13.9	26.8
	4	7.3	0.415	0.192	75.0	4.27	81.78	64.2	10.3	26.0
	5	7.8	0.386	0.183	81.0	4.01	87.80	62.5	10.4	25.9

(b) *Potassium*.

Considerable discussion has arisen with regard to the relation between the potassium and calcium nutrition of the plant. Ehrenberg (7), Fonder (9),

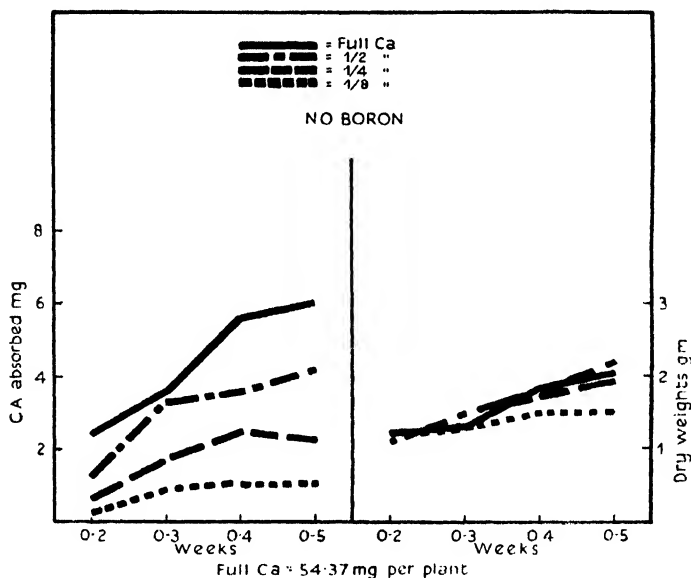


FIG. 7. Relation between calcium absorption, dry matter production, and the age of *V. faba* when supplied with different amounts of calcium. Solutions not renewed. 1931.

Lipman, Blair, and Prince (19), Sewell and Latshaw (33), and Lagatu and Maume (16) found a reduction in the quantity of potassium absorbed when large amounts of calcium were present in the soil, while Reed and Haas (32), and Newton (24), working with culture solutions, have recorded a similar inverse relationship between the two elements, the potassium content of the plant increasing if the supply of calcium was deficient. Gile and Ageton (10), and Pfeiffer and Rippel (27), on the other hand, did not find any inverse relationship to hold, and Plummer (29) even obtained an increase in potassium removed by soy beans when the soil was limed. This, however, he considered due to improved soil conditions rather than to any direct influence of the lime on the potash uptake. Colby (5) also failed to obtain any increase in potassium content in plants grown with very depleted supplies of calcium, a result he attributed to the fact that such large amounts of potassium were already present in the plant that a further rise could hardly be expected. This suggestion may well reconcile the apparently conflicting results referred to above.

In view of the interest attached to the potassium-calcium ratio, some

potassium estimations were carried out on the ash of plants in the course of the present work, four-week-old plants grown in unrenewed solutions being selected as likely to give the best-defined results. The cobaltinitrite

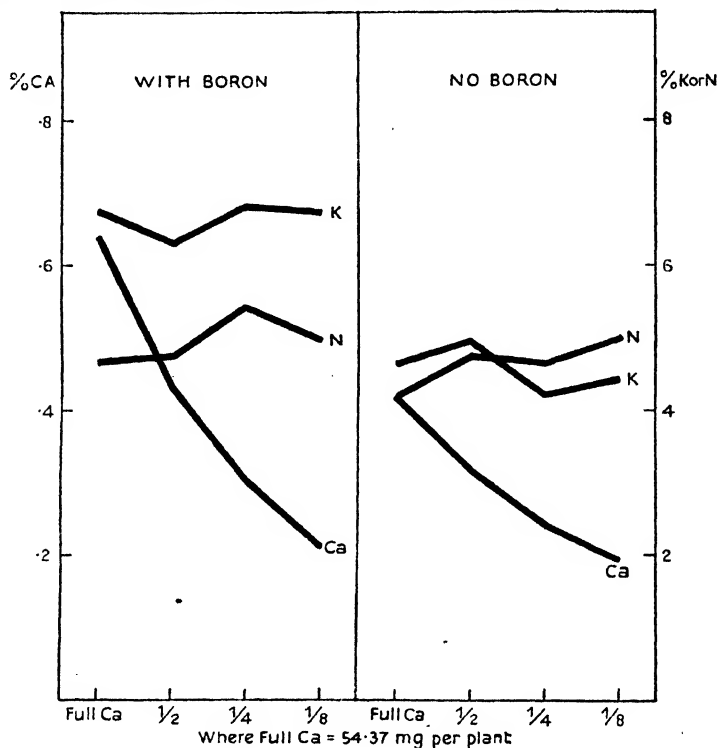


FIG. 8. Relation between the percentage calcium, potassium, and nitrogen content of *V. faba* with the quantity of calcium supplied. Age four weeks. 1931.

volumetric method was used as described by Milne (23), and the values given are the mean of closely agreeing duplicates. As in the case of the nitrogen figures, terms of content, and not absorption, have been used throughout in comparing the potash and calcium figures.

(1) *Boron supplied.*

Under full nutrient conditions the potassium, as well as the calcium content of the plants, increased with advancing age, but as the rate of increase was somewhat larger in the case of the latter element, a slight fall in the K/Ca ratio was found with time (Table XII) as Fonder (9) also described. The drop, however, was much less marked than was the case with the N/Ca ratio. No question of shortage of available potash comes into play here, as even after five weeks' growth only 50 per cent. of the potash supplied had been absorbed. In the presence of a deficient calcium

supply (one-eighth full quantity, or 6.80 mg. calcium per plant), the uptake of potassium was amazingly little reduced, in fact the dry weight tended to be more adversely affected as the slightly higher value for the percentage potassium in the five-week-old plants shows (Table XII). In consequence the K/Ca ratio showed a rise instead of a fall where the calcium supply was deficient. No definite correlation was found between the calcium supplied and the amount of potash absorbed by four-week-old plants (Figs. 4 and 8), though there is some suggestion that an inverse relationship was beginning to make its appearance after a further weeks' growth, as by then the percentage of potassium was slightly higher in the plants grown in the medium deficient in calcium than in the complete nutrient solution. Colby's explanation (to which reference has already been made) may be applicable here as the salt content of the ash of plants grown in water cultures is high, but Lundegårdh's results with wheat (21, Fig. 41) suggest that within the limits of the concentration of calcium used in the present experiments, no very close association between the calcium provided and the potash absorbed is to be expected, and that only with larger concentrations of calcium would the inverse relationship between the two elements be at all clearly defined. A further point of interest arises here in connexion with Lundegårdh's work, as he found that on an average the potassium content of wheat was approximately ten times greater than the calcium content, a figure in close agreement with the present results, in spite of the fact that the latter were obtained with such a widely differing species as *Vicia faba*. Though no statistically significant correlation was found, the potash content followed the dry matter curve very closely. As a result the curves for nitrogen and potash contents were very similar, neither showing any relationship to the quantity of calcium in the solution or in the plant (Fig. 8).

(2) *No boron.*

The results obtained were similar to those already described for nitrogen. If the calcium in the nutrient solution were plentiful, both the calcium and potash contents of the plant rose with advancing age, at least up to the fourth week, though the increase was much less marked than where boron was supplied. The K/Ca ratio, however, showed no tendency to fall after the third week, a fact which suggests that the lack of boron, while reducing the absorption of both elements, was exerting the greater influence on the uptake of calcium (Table XIV). If the calcium supply was deficient, the K/Ca ratio failed to rise with time after the third week as had occurred where boron were present, though as would be expected, the level attained was much higher than where the quantity of calcium supplied was larger (Table XIV). A lack of boron, therefore, was reducing the absorbing power of the plant for potassium as well as for calcium as

has been stated above, and further, affected the uptake of potash more than the uptake of nitrogen. The potassium content of four-week-old plants showed little, if any, correlation with the amount of calcium supplied in the solution (Fig. 6), and what trace of relationship is to be seen is of a direct rather than an inverse nature, so the results are in general agreement with those from plants grown with boron.

A deficiency of boron, therefore, tended to reduce the uptake of calcium more than that of nitrogen or potash, which would seem to indicate a closer relationship between boron and calcium, than between boron and nitrogen, or boron and potassium. At the same time it must be borne in mind that nitrogen and potassium are generally most vigorously absorbed during the early stages of growth, whereas calcium is still freely taken up at later stages of development just when the effects of any deficiency of boron have reached their full importance. Further, a lack of boron reduces the development of the root more seriously than that of the shoot, so that a diminished capacity for absorption of all nutrients inevitably results when boron is withheld.

III. DISCUSSION.

A consideration of the foregoing results shows that the quantity of calcium a plant absorbs is influenced by a number of factors. The age of the plant is a matter of importance, for in general the absorption increases with age up to quite a late phase of development, though some periodicity in uptake is also evinced. In this connexion the necessity for carrying on experiments for sufficiently long periods, if a true picture of the events is to be secured, is clearly brought out. Another factor upon which the extent of the absorption of calcium depends is the quantity of the calcium supplied, and although certain concentrations appear to be more economically utilized than others, the uptake is approximately proportional to the amount supplied. Based upon dry matter production, the absorption of calcium is greater from a pure solution of calcium chloride than from complete nutrients, and a similarly reduced uptake is obtained when the nutrient medium is frequently renewed, that is to say, the absorption of calcium is affected by the presence and quantity of other elements.

The question as to whether or not any association exists between boron and calcium uptake must, therefore, be considered under a variety of conditions. The approach to the problem, however, is rendered particularly difficult by the fact that death ensues in the absence of boron, and in consequence all metabolic processes receive a check, and any condition which hastens or retards the onset of the boron deficiency symptoms will inevitably alter the absorbing capacity of the plant. Some points, however, stand out distinctly. In the first place certain main results hold good irrespective of whether boron is present or not, the differences being those

of degree only. For example, a proportional relationship between the calcium supplied and absorbed, and an increased uptake as the plant developed is found in both cases, though the quantities absorbed in the absence of boron are considerably the smaller. In the second place, other results are dependent upon whether the plants are grown with or without boron, but such differences can almost always be traced to the effect of the nutritive conditions on the rapidity with which boron deficiency symptoms appear. An example of this is found in the greater absorption of calcium from unrenewed than from frequently renewed solutions when boron is present, whereas in the absence of boron the reverse is the case. This apparently direct association between the reduction in calcium uptake and the lack of boron has, therefore, to be viewed in the light of the plants' capacity to absorb other elements, i.e. allowance must be made for the general reduced vitality of the plant in the absence of boron, before any conclusions can be drawn. Both the potassium and the nitrogen contents are much less affected by the absence of boron than is the calcium content, so that an affinity between boron and calcium is certainly suggested. The data which point to such an association most strongly, however, are those obtained firstly, from comparisons of plants grown in complete nutrients with those in a pure solution of calcium chloride, and secondly, between those grown in unrenewed and frequently renewed solutions. In both cases the conditions which delayed the onset of the boron deficiency symptoms (i.e. the presence of complete nutrients and their frequent renewal respectively) also inhibited the uptake of calcium in the presence of boron, facts which certainly point to an affinity between the two elements, for if boron were needed for the uptake of calcium, and its supply limited to that originally provided by the seed, any condition which would tend to increase the absorption of calcium, such as the absence of other nutrients, would also hasten the appearance of boron deficiency symptoms. It is, therefore, of particular interest that somewhat similar views should have been expressed in two recent publications, though the question was approached from rather a different aspect than has been the case in the present paper. Haas (12), in a study of the effect of toxic quantities of boron on fruit trees, showed that the presence of excess of this element resulted in a decreased absorption of calcium by citrus and walnut. Bobko and Belvoussev (2), on the other hand, found that injury to sugar beet induced by excessive calcium in the soil could be corrected, and in fact the calcium turned to good account, by the application of small quantities of boron. Excess of either element, therefore, affected the need of the plant for the other. At the same time, it has to be borne in mind that although the behaviour of elements needed in such small quantities as boron may well differ from those required in larger amounts, yet in general no one element bears a unique relationship to one other element, excess or

deficiency of any individual nutrient usually bringing about a number of changes, both chemical and physiological, owing to the displacement of the balance between all the components of the plant's food.

The evidence so far obtained, though insufficient to provide any definite proof of an association between boron and calcium, does, however, suggest that a relationship of some sort exists between them, the exact nature of which is as yet undetermined.

IV. SUMMARY.

1. Over a nine-week period the quantity of calcium absorbed per week by *Vicia faba* increased up to the seventh or fourth week respectively, according as to whether or not a trace of boron was present, indications of periodicity in uptake being obtained in the former case.

2. Less calcium was absorbed from solutions renewed at weekly or fortnightly intervals, than from unrenewed solutions when boron was present, but the reverse was the case if boron were not provided. This difference is attributed to the fact that renewal of the solution delays the appearance of the boron deficiency symptoms, and thus prolongs the absorbing capacity of the plant.

3. The quantity of calcium absorbed was approximately proportional to the calcium supplied, irrespective of the presence or absence of boron, although the total calcium taken up was much reduced under the latter condition.

4. No correlation was found between the calcium supplied and the nitrogen or potash content of the plant, both the latter showing a closer affinity with the production of dry matter.

5. Under full nutrient conditions the N/Ca and K/Ca ratios in the plant fell as its age increased, the fall being more marked in the presence than in the absence of boron. A lack of boron, therefore, reduced the uptake of calcium more than that of nitrogen or potash.

6. In the presence of boron, the calcium absorbed per unit dry matter produced was higher from a pure solution of calcium chloride, than from a complete nutrient medium containing a similar quantity of calcium. In the absence of boron, death ensued the more rapidly in the plants grown in the single salt solution, so that the presence of other nutrients apparently increased the requirement of the plant for both calcium and boron. Although the evidence is not conclusive, indications of an association between boron and calcium were, therefore, obtained.

In conclusion, thanks are due to Mr. R. G. Warren for his helpful advice with regard to the chemical methods employed, and to Professor Lundegårdh for his generosity in placing his valuable experience and laboratory facilities at the writer's disposal.

LITERATURE CITED.

1. BRENCHELEY, W. E., and WARINGTON, K. : The Rôle of Boron in the Growth of Plants. *Ann. Bot.*, xli. 167-87, 1927.
2. BOBKO, E. V., and BELVOUSSOV, M. A. : Importance du Bore pour la Betterave à Sucre. *Ann. Agron.*, iii. 493-504, 1923.
3. BURD, J. S. : Rate of Absorption of Soil Constituents at Successive Stages of Growth. *Journ. Agric. Res.*, xviii. 51-72, 1919.
4. CHAPMAN, H. D. : The Precipitation of Calcium Oxalate in the Presence of Iron, Aluminium, Titanium, Manganese, Magnesium and Phosphates, with Special Reference to the Determination of Total Soil Calcium. *Soil Sci.*, xxvi. 479-86, 1928.
5. COLBY, H. L. : Seasonal Absorption of Nutrient Salts by the French Prune Grown in Solution Culture. *Plant Phys.*, viii. 1-34, 1933.
6. DAY, D. : Some Effects of Calcium Deficiency in *Pisum sativum*. *Plant Phys.*, iv. 492-506, 1929.
7. EHRENBERG, P. : Das Kalk-Kali Gesetz. *Land. Jahrb.*, liv. 1-159, 1919.
8. FONDER, J. F. : The Relationship of Soil Type to the Calcium and Magnesium Content of Green Bean Stems and Leaves and of their Expressed Juice. *Soil Sci.*, xxvii. 415-31, 1929.
9. ——— : Variations in Potassium Content of Alfalfa due to Stage of Growth and Soil Type and to the Relationship of Potassium and Calcium in Plants Grown upon Different Soil Types. *Journ. Amer. Soc. Agron.*, xxi. 732-50, 1929.
10. GILR, P. L., and AGETON, C. N. : The Effect of Strongly Calcareous Soils on the Growth and Ash Composition of Certain Plants. *Porto Rico Agric. Expt. Sta. Bull.* 16, 1914.
11. GINSBURG, J. M., and SHIVE, J. W. : The Influence of Calcium and Nitrogen on the Protein Content of the Soybean Plant. *Soil Sci.*, xxii. 175-97, 1926.
12. HAAS, A. R. C. : Toxic Effect of Boron on Fruit Trees. *Bot. Gaz.*, lxxxviii. 113-31, 1929.
13. HARTMANN, C., and POWERS, W. L. : The Crop Producing Power of Limited Quantities of 'Essential' Plant Nutrient. *Soil Sci.*, xxv. 37-77, 1928.
14. HOLTZ, H. F. : Effect of Calcium and Phosphorus Content of Various Soil Series in Western Washington upon the Calcium and Phosphorus Composition of Oats, Red Clover and White Clover. *Wash. Agric. Expt. Sta. Bull.*, 243, 1930.
15. JACOBSON, H. G. M., and SWANBACK, T. R. : Relative Influence of Nitrate and Ammoniacal Nitrogen upon Intake of Calcium by Tobacco Plants. *Plant Phys.*, viii. 340-2, 1933.
16. LAGATU, H., and MAUMÉ, L. : Antagonisme du Calcaire, à l'Égard de l'Absorption de la Potasse par la Vigne. *Com. Cong. Internat. du Vin et du Pin Maritime. Bordeaux*, 1928.
17. LARSON, H. W. : The Relation of the Concentration of Calcium Ion Required by Alfalfa to the Amount Present in Soil Solution. *Soil Sci.*, xxv. 399-408, 1928.
18. LIPMAN, J. G., and BLAIR, A. W. : Yield and Nitrogen Content of Soybeans as Influenced by Lime. *Soil Sci.*, iv. 71-7, 1917.
19. ———, and PRINCE, A. L. : The Effect of Lime and Fertilizers on the Potash Content of Soil and Crop. *Internat. Rev. Sci. Prac. Agric.*, iv. 546-53, 1926.
20. LUNDEGÅRDH, H. : Die quantitative Spektralanalyse der Elemente. *Fischer, Jena*, 1929.
21. ——— : Die Nährstoffaufnahme der Pflanze. *Fischer, Jena*, 1932.
22. McMURTREY, J. E. : Distinctive Effects of the Deficiency of Certain Essential Elements on the Growth of Tobacco Plants in Solution Cultures. *U.S. Dept. Agric. Tech. Bull.* 340, 1933.
23. MILNE, G. : The Cobaltinitrite (Volumetric) Method of Estimating Potassium in Soil Extracts. *Journ. Agric. Sci.*, xix. 541-52, 1929.
24. NEWTON, J. D. : A Comparison of the Absorption of Inorganic Elements and of the Buffer Systems of Legumes and Non-legumes and its Bearing upon Existing Theories. *Soil Sci.* xv. 181-204, 1923.
25. NIGHTINGALE, G. T., et al. : Effects of Calcium Deficiency on Nitrate Absorption and on Metabolism in Tomato. *Plant Phys.*, vi. 603-35, 1931.
26. PARKER, F. W., and TUROG, E. : The Relation between the Calcium and the Nitrogen Content of Plants and the Function of Calcium. *Soil Sci.*, x. 49-60, 1920.

776 *Warington.—Absorption of Calcium from Nutrient Solutions.*

27. PFEIFFER, T., and RIPPET, A.: Der Einfluss von Kalk und Magnesia auf das Wachstum der Pflanzen. *Journ. f. Landw.*, lxxiii. 5-39, 1920.
28. PHILIPSON, C.: Growth and Absorption by Oats in Relation to Varying Concentrations of Calcium and Sodium in the Nutrient Solution. *Svensk. Bot. Tidskr.*, xxiv. 155-256, 1930.
29. PLUMMER, J. K.: Availability of Potash in Some Common Soil-Forming Minerals. Effect of Lime upon Potash Absorption by Different Crops. *Journ. Agric. Res.*, xiv. 297-315, 1918.
30. REDFERN, G. M.: On the Absorption of Ions by the Roots of Living Plants. I. The Absorption of the Ions of Calcium Chloride by Peas and Maize. *Ann. Bot.*, xxxvi. 167-74, 1922.
31. REED, H. S., and HAAS, A. R. C.: Effect of Sodium Chloride and Calcium Chloride upon Growth and Composition of Young Orange Trees. *Calif. Agric. Exp. Sta. Tech. Paper*, iv. 1923.
32. —————: Studies on the Effects of Sodium, Potassium and Calcium on Young Orange Trees. *Calif. Agric. Exp. Sta. Tech. Paper*, xi. 1923.
33. SEWELL, M. C., and LATSHAW, M. L.: The Effect of Lime, Superphosphate and Potash on Reaction of Soil and Growth and Composition of Alfalfa. *Journ. Amer. Soc. Agron.*, xxiii. 799-814, 1931.
34. SHEDD, O. M.: Effect of Certain Calcium Compounds and Other Substances on the Yield and Calcium Content of Some Crops. *Soil Sci.*, xiv. 233-46, 1922.
35. WARINGTON, K.: The Influence of Length of Day on the Response of Plants to Boron. *Ann. Bot.*, xlvii. 429-57, 1933.
36. WAYNICK, D. D.: The Chemical Composition of the Plant as further Proof of the Close Relation between Antagonism and Cell Permeability. *Univ. Calif. Pub. Agric. Sci.*, iii. 135-242, 1918.

THE EFFECT OF SODIUM NITRATE ON THE GROWTH AND NITROGEN CONTENT OF A LUCERNE AND GRASS MIXTURE.

BY H. G. THORNTON AND HUGH NICOL.

(*Department of Bacteriology, Rothamsted Experimental Station, Harpenden, Herts.*)

(With Twelve Text-figures.)

I. INTRODUCTION.

THE influence of mineral nitrogenous manuring upon an association of leguminous and other plants is of considerable agricultural importance. The problem bears on the manuring of grass land, forage mixtures, temporary leys, and of such legumes as clover and lucerne where these are sown in a cereal cover crop. The practice of applying a small dressing of mineral nitrogen to a leguminous crop grown by itself is often held to be beneficial, on the ground that it gives the legume a good start. Thus, in a rotation experiment carried out at Rothamsted, sulphate of ammonia increased the yield of pure clover to an appreciable extent (see *Rothamsted Experimental Station Report for 1930*, pp. 37, 38). Too often, however, a legume, though sown by itself, actually grows in association with a flora of weeds, so that here, too, nitrogenous manuring may be acting upon a mixed flora. Where a legume is being grown, intentionally or otherwise, in association with a non-legume, the response of the crop is complicated by the introduction of the factor of competition.

Numerous field experiments with permanent grass, temporary leys, and forage mixtures, have shown that nitrogen dressings may stimulate the non-leguminous plants to such a degree that they will check the growth of the legumes. In consequence of their effect in diminishing the percentage of legumes, nitrogenous manures often fail to increase the content of protein of the crop. A good example of this was afforded by a forage mixture experiment (oats or barley, with vetches or peas) on arable land, commenced at Rothamsted in 1930. In its first year this experiment gave the results tabulated below (see *Rothamsted Reports for 1930, 1931, and 1932*):

Nitrogen added (cwt. per acre)	0	0.2	0.4
Yields of dry matter (cwt. per acre)	25.3	31.8	35.8
Percentage of protein in crop	11.7	9.6	8.6
Nitrogen in the crop (cwt. per acre)	0.42	0.44	0.44

The effect of mineral nitrogen on mixtures of legumes and non-legumes has been very little studied under the better-controlled conditions of pot experiments. Remy and Vasters(3), however, made a complicated pot experiment containing non-legumes with two legumes, and applied nitrogen at four different levels, all of them high. The relation of nitrogen applied to that recovered in the crop is shown in Fig. 1. Only the highest nitrogen dose increased the nitrogen content of the crop; with

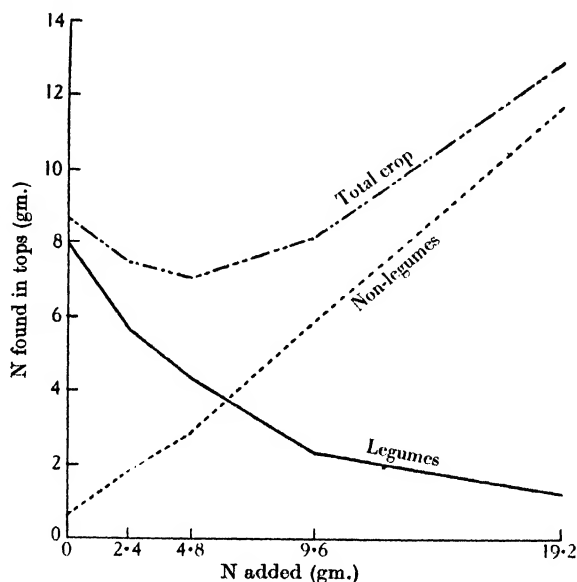


Fig. 1. From data of Remy and Vasters (3).

the other dressings the better growth of non-legumes was offset by decreases in the legumes.

Evidence from such work, however, does not enable us to distinguish, in the legume, the effects of increased competition from those due to possible directly repressive action of the applied nitrogen. In order, firstly, to estimate the relative importance of these two effects, and, secondly, to determine whether the non-legume sets up competition above or below ground, a pot experiment was carried out during the summer of 1931. In this experiment lucerne was grown alone and with grass, in sand to which three different dressings of sodium nitrate were applied.

II. PLAN OF THE EXPERIMENT.

The experiment was performed in glazed earthenware pots, each containing about 20 kg. of sand having an initial water content of 2 per cent. 10 gm. of precipitated chalk was well mixed with the sand before filling the pots, and to each pot was given 500 ml. of the following plant nutrient solution, made with the usual hydrated salts:

0.5 gm. K_2HPO_4	0.5 gm. NaCl
0.5 „ KH_2PO_4	0.5 „ $CaSO_4$
0.5 „ $MgSO_4$	0.04 „ $FeCl_3$

The pots were divided into the following series each containing twelve parallel pots:

Series	Nitrate manuring	Sowing
A	+0.33 gm. $NaNO_3$	Lucerne alone
B	+1.0 „ „	
C	+3.0 „ „	
AA	+0.33 „ „	Lucerne and Italian rye grass
BB	+1.0 „ „	
CC	+3.0 „ „	

All the nitrate was added in one dose in solution before sowing. The pots were sown on April 25 with 200 lucerne seeds per pot and series AA, BB and CC with 240 seeds of Italian rye grass as well. The lucerne seed was inoculated with an efficient strain of the lucerne nodule bacteria using the technique described by Thornton(4). The pots were arranged in the greenhouse in twelve blocks within each of which the treatments were randomised. They were weighed at the commencement of the experiment and watered with rain water as required to bring them to their initial weight. The plants were thinned out on May 13-15, leaving twenty lucerne plants and, in series AA, BB and CC, twenty grass plants per pot.

III. EARLY GROWTH.

A count of seedlings made on May 5 showed that the higher nitrate dressings significantly delayed germination and early growth of both the lucerne and the grass. The effect on the lucerne was still shown by a leaf count made on May 19. A second leaf count made on May 30 showed that by this date the number of leaves on plants receiving 1.0 gm. of sodium nitrate was no longer significantly lower than on those receiving 0.33 gm. The effect of grass competition was not yet apparent. During June, however, the grass began to show marked differences both in height and number of shoots in the three series. In the AA series it was very weak

and etiolated. In series BB and CC it was green, and in CC the growth was much the strongest. The differences were apparent up to the end of the experiment. By June 30 the growth of the lucerne was depressed by the presence of grass but was not significantly affected by the nitrate dressing (Table I). The directly harmful effects of the higher nitrate dressings upon the lucerne, noted in the early growth, were thus no longer detectable by this date.

Table I. *Early growth of the lucerne grown with and without grass.*

Series	Treatment	Mean number of lucerne leaves per pot,	Mean number of lucerne leaves per plant,	Mean height of lucerne plant
		May 19	May 30	June 30
A	+0.33 gm. NaNO_3	42.0 ± 1.2	3.5 ± 0.08	43 cm. ± 2.4
B	+1.0 " "	39.9 ± 1.38	3.5 ± 0.12	44 " ± 2.2
C	+3.0 " "	35.7 ± 1.60	2.8 ± 0.13	39 " ± 2.7
AA	+0.33 " "	42.5 ± 1.33	3.4 ± 0.11	34 " ± 1.7
BB	+1.0 " "	39.2 ± 1.18	3.2 ± 0.11	30 " ± 1.2
CC	+3.0 " "	31.7 ± 1.39	2.8 ± 0.08	31 " ± 1.5

IV. REAPING OF THE CROP.

Reapings were made on June 25, July 27, August 28 and October 1, three pots in each series being reaped on each date. The reaping was carried out as follows. The pots to be reaped were left unwatered for several days till the sand became dry. The whole contents were then turned out and the plants lifted out of the sand and gently shaken. It was found possible by this means to separate the roots of the grass and of the lucerne. The sand was then passed through a sieve to catch such fragments of fine grass roots as were left in it. All roots were then washed free from sand and the nodules on the lucerne roots were counted. Tops and roots of both lucerne and grass were separated, weighed in an air-dry condition, and analysed for nitrogen by the Kjeldahl method. The nitrogen estimations were performed by the Chemistry Department of this Station.

Each point shown in the graphs which follow represents the mean of values obtained from three parallel pots.

V. EFFECT OF NITRATE DRESSINGS ON LUCERNE GROWN ALONE.

In series A, B and C, where lucerne was grown alone, the variation in dose of nitrate produced no significant effect upon the yield or the nitrogen content of the tops (Fig. 2). The highest dressing significantly reduced the root growth in the last three months (Fig. 3).

The number of nodules developed by the time of the first reaping in

June was considerably reduced by the highest nitrate dose, but by July the nodule numbers in the three series were not significantly different (Fig. 4). In August and especially in September there was an actual increase in nodule numbers with quantity of nitrate supplied. This result in the last two reapings differs from the usual finding that nodule numbers are reduced by nitrate. In the present experiment rather small nitrate

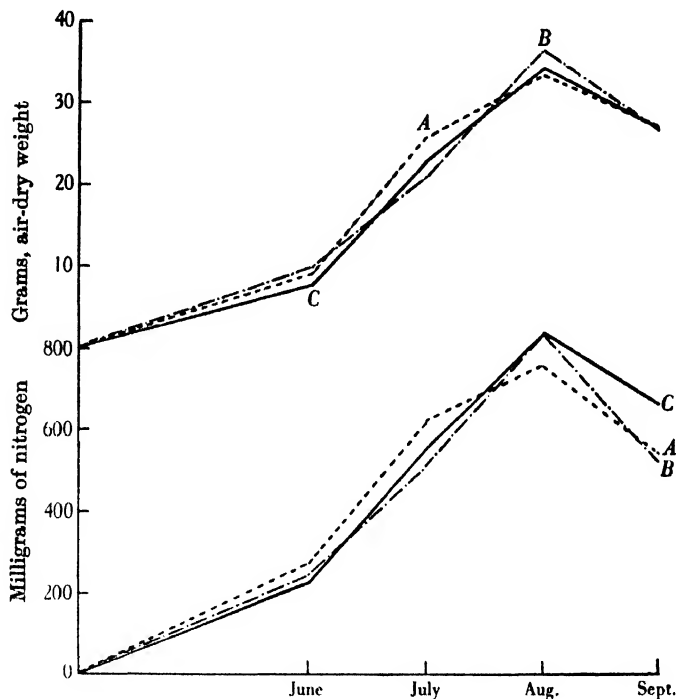


Fig. 2. Lucerne grown alone. Yields and nitrogen content of tops.

applications were made in April, so that by August the nitrate concentration in all three series may well have been so greatly reduced as no longer to check infection by the nodule bacteria. It does not seem profitable to discuss the reason for the actual increase in nodule numbers with nitrate, since the data do not enable us to test the possible causes that suggest themselves. The greater number of nodules in series B and C was not accompanied by any increase in nitrogen content of the plants, so that less nitrogen must have been fixed per nodule with the higher nitrate applications.

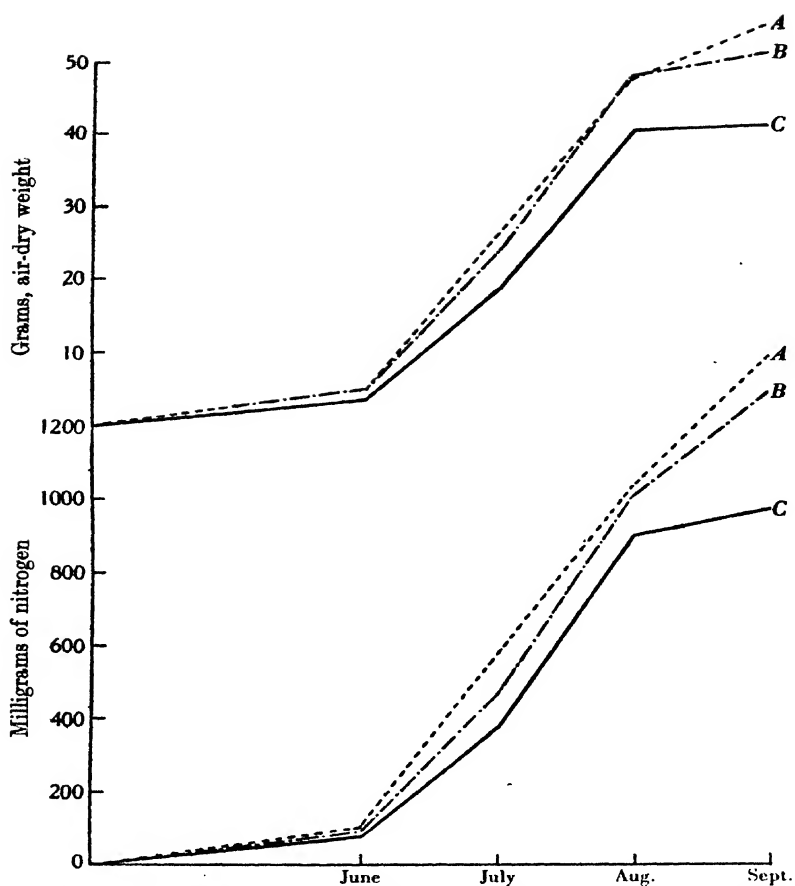


Fig. 3. Lucerne grown alone. Yields and nitrogen content of roots.

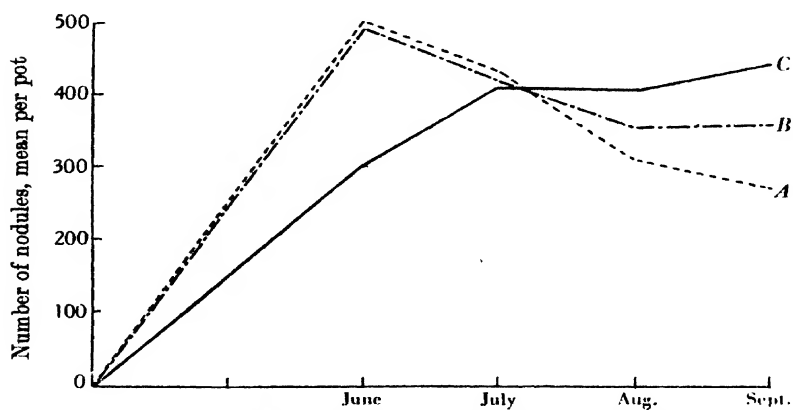


Fig. 4. Lucerne grown alone. Nodule numbers.

VI. EFFECT OF NITRATE DRESSINGS UPON THE LUCERNE-RYE GRASS ASSOCIATION.

In series AA, BB and CC, the growth and nitrogen content of lucerne, grown in association with grass, were inversely related to the dose of nitrate supplied (Figs. 5 and 6). Series CC, receiving 3 gm. of NaNO_3 , gave a yield and nitrogen content both of lucerne tops and roots that was significantly lower than the other two series from July onwards. Series BB, receiving 1 gm. of nitrate, gave a significantly lower yield and

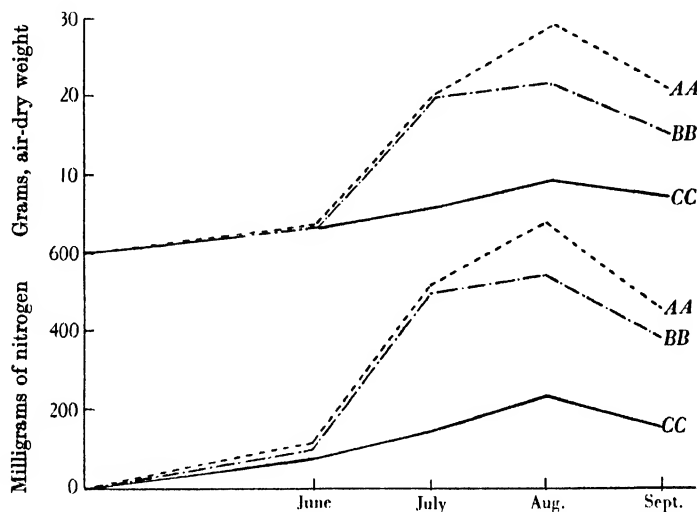


Fig. 5. Lucerne grown with grass. Yields and nitrogen content of lucerne tops.

nitrogen content of lucerne tops and roots than series AA in August and September. The nodule numbers in July, August and September, like the root weights, showed a significant inverse relation to amounts of nitrate added (Fig. 7).

Growth of the grass was markedly increased by the two larger nitrate dressings (Fig. 8). It seems clear that the harmful effect of these dressings upon lucerne growing with the grass was due to competition. The lucerne grew so much taller than the grass even in series CC, that it can scarcely have been weakened appreciably by shading. It seems probable, therefore, that the lucerne was affected by root competition. This probability is supported by the fact that the total lucerne dry matter in July, August and September was a linear decreasing function of the grass root weights, so that for every gram increase in grass roots the lucerne decreased by

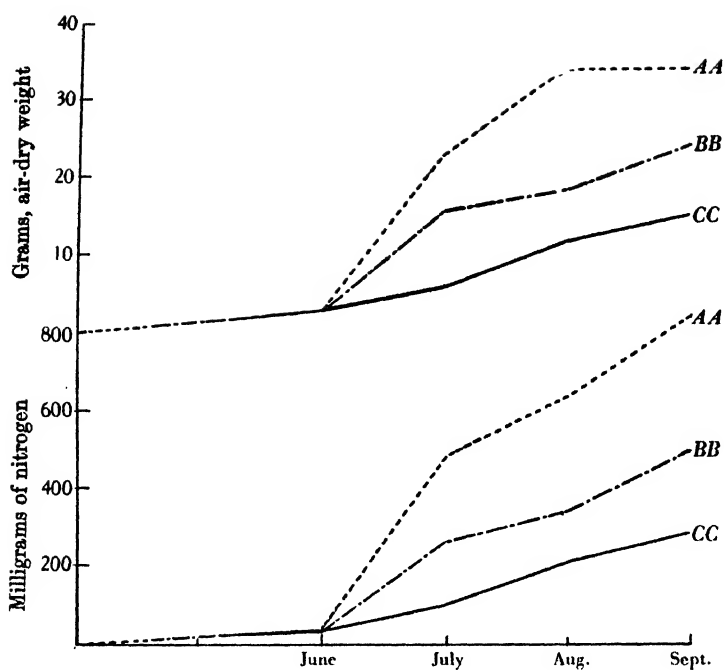


Fig. 6. Lucerne grown with grass. Yields and nitrogen content of lucerne roots.

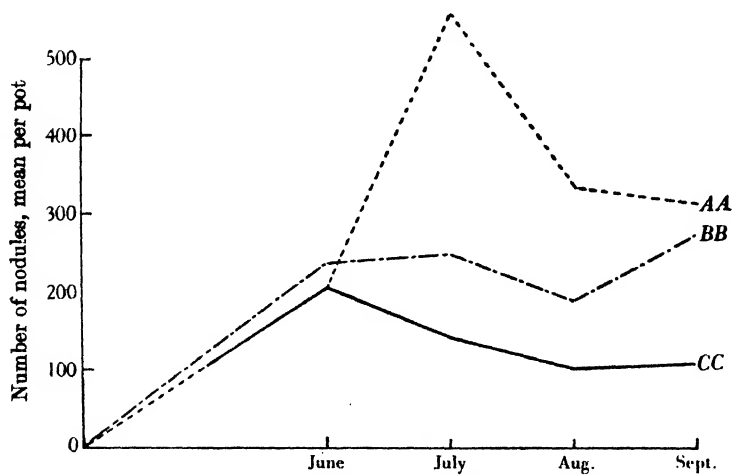


Fig. 7. Lucerne grown with grass. Nodule numbers.

about 0.8 gm. On the other hand the lucerne dry matter was less clearly related to that of the grass tops (Fig. 9).

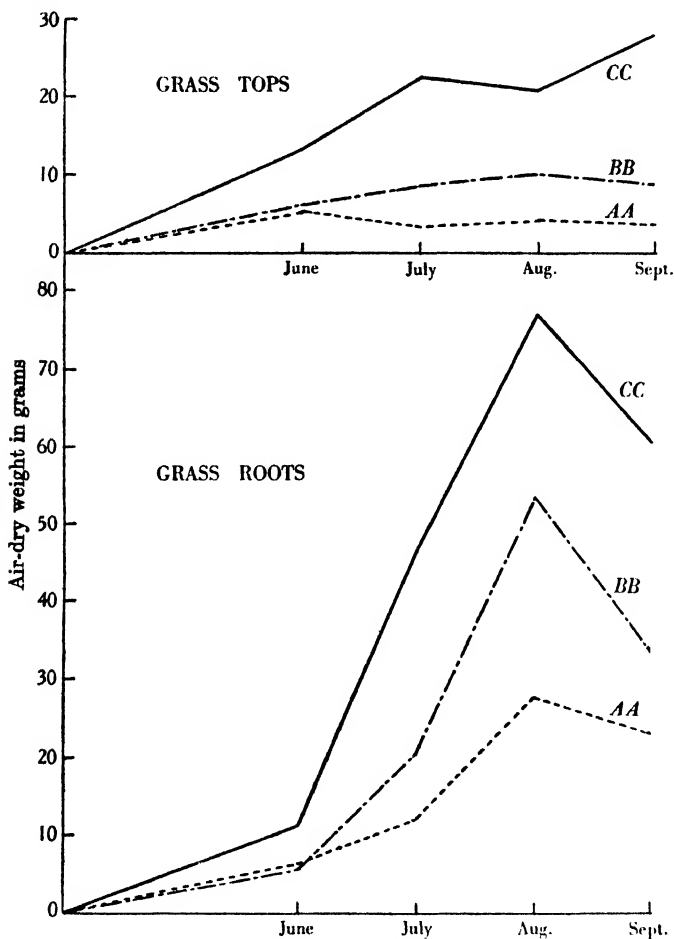


Fig. 8. Yields of grass growing with lucerne.

VII. ABSORPTION BY THE GRASS OF NITROGEN FIXED BY THE LUCERNE.

In series AA and BB, the quantities of nitrogen contained in the grass towards the end of the experiment greatly exceeded the doses of nitrate nitrogen added (Fig. 10). In series AA, where 55 mg. of nitrate nitrogen were added to each pot, the grass was found to contain 132 mg. of nitrogen per pot in July and 300 mg. in August. In series BB, where 163 mg. of nitrate nitrogen were supplied, the grass contained 188 mg. of

N in July and 389 mg. in August. Thus, even if the whole of the nitrate nitrogen had been taken up by the grass and none by the lucerne, con-

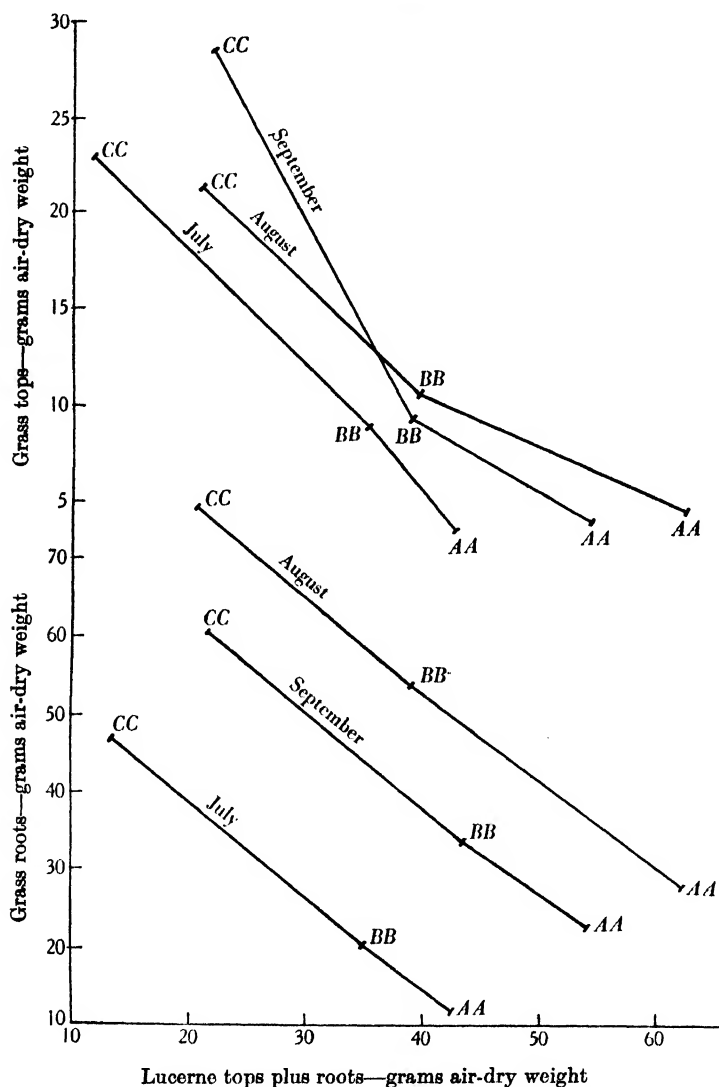


Fig. 9. Lucerne air-dry weights plotted against grass tops and roots. (AA, BB and CC.)

siderable additional quantities of nitrogen were gained by the grass. The bulk of this extra nitrogen must have been derived from nitrogen fixed by the lucerne. The fact that grass can obtain nitrogen from lucerne

within 3 months of sowing suggests the excretion of nitrogen compounds by the latter, since no decay of the lucerne roots was apparent.

Evidence from numerous field experiments (*e.g.* Dorsey (5), Lyon (6)) in which growth of a non-legume population was assisted by the presence of a legume has suggested an excretion of nitrogenous material from the roots of the latter (*cf.* J. G. Lipman (1, 2)). The mode of this transfer from legume to non-legume has been but little investigated, and until quite recently its existence has been inferred rather than demonstrated. Stallings (7) postulated such a transfer of a water-soluble nitrogen com-

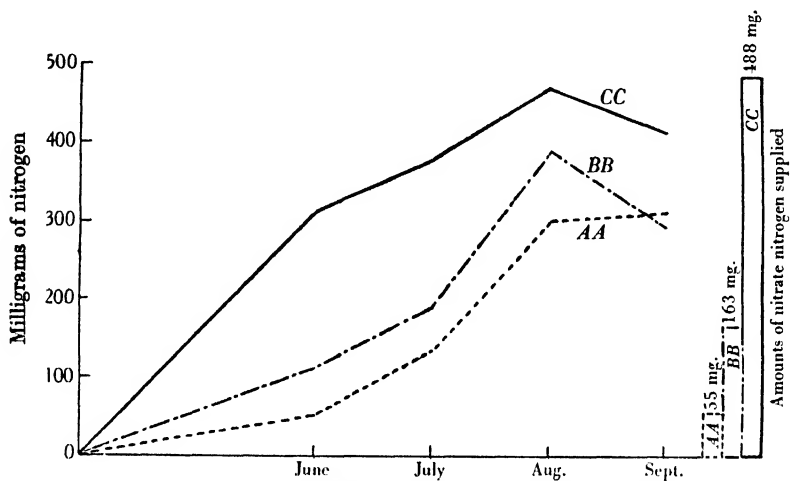


Fig. 10. Nitrogen content of grass tops and roots together.

pound—possibly ammonia. Joshi (8) recorded an effect upon non-legumes supposed to be due to secretions of legume nodule bacteria.

The question has been investigated in some detail by Virtanen and his co-workers (9, 10, 11, 12, 13, 14). They grew peas, inoculated but otherwise sterile, in sand, and found that the sand contained an amount of combined nitrogen of the same order as did the peas themselves. This nitrogen, which was largely in the form of free amino-acids (9), could be profitably utilised by not more than two oat plants grown in association with each pea. See review by Nicol (16).

VIII. DISCUSSION.

The doses of nitrate applied in this experiment were small, representing in the three series 2.75, 8.15, and 24.4 parts of nitrogen per million of sand, respectively. The sand itself contained inappreciable

amounts of combined nitrogen. These applications did not materially affect the crop obtained when lucerne was grown alone.

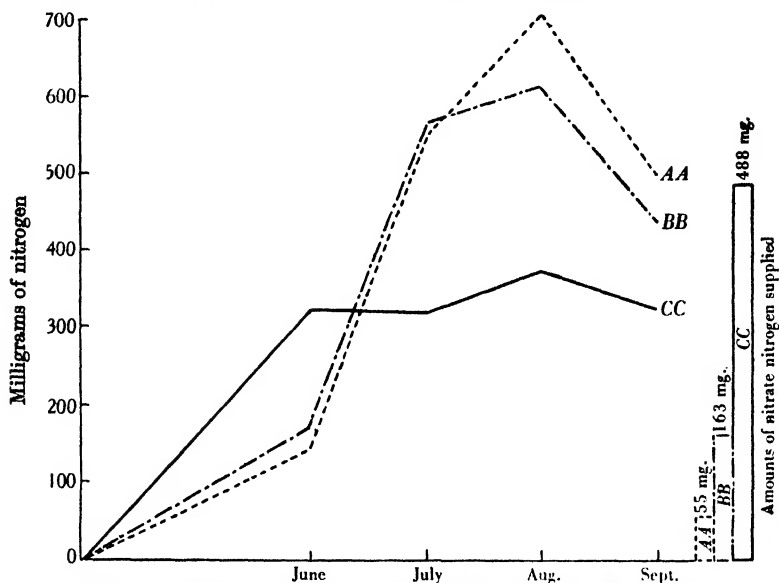


Fig. 11. Nitrogen content of lucerne tops and grass tops together.

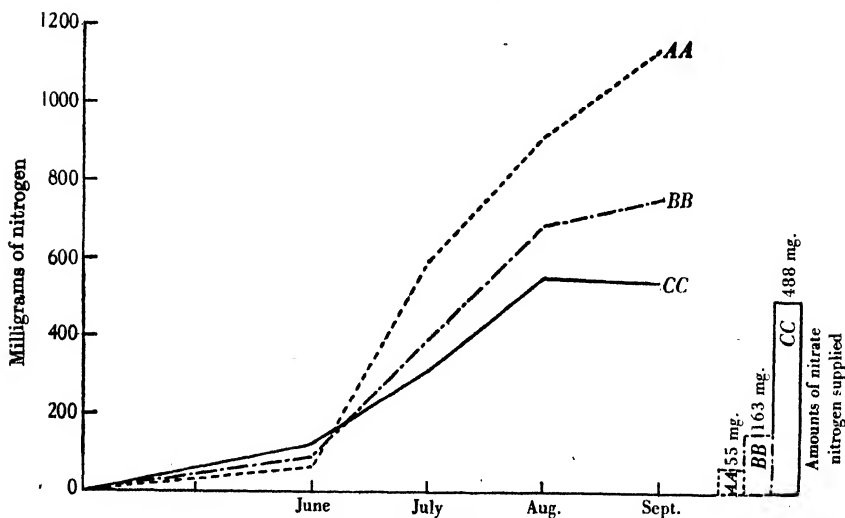


Fig. 12. Nitrogen contents of lucerne roots and grass roots together.

When the lucerne was grown with Italian rye grass, however, the two higher dressings considerably reduced the nitrogen content of the crop

obtained from the combined lucerne and grass (Fig. 11). They also had a similar effect in reducing the quantity of nitrogen stored in the roots (Fig. 12).

The results of the experiment thus show that dressings of nitrate when applied to lucerne that is grown together with a non-legume may have a harmful effect, reducing both the protein content of the crop and also the quantity of combined nitrogen left in the soil to benefit the succeeding crop.

IX. SUMMARY AND ABSTRACT.

1. Inoculated lucerne was grown alone and in association with Italian rye grass, in pots of sand watered with food solution and given three different doses of sodium nitrate.

2. The dose of nitrate did not affect the dry weight or nitrogen content of lucerne when grown alone, save that the highest dose checked the root growth somewhat.

3. When lucerne and Italian rye grass were grown in association, the growth of the grass varied directly with the dose of nitrate applied, and the growth of the lucerne varied inversely to it. Checking of the lucerne growth was probably due to root competition with the grass.

4. The nitrogen contents of the combined lucerne and grass tops and that of the combined roots were also inversely related to the quantity of nitrate applied.

5. There was evidence that within 3 months of sowing the grass had obtained nitrogen fixed by the lucerne nodules.

ACKNOWLEDGMENT.

The authors' thanks are gladly accorded to Miss Sheila Arnold for her assistance in making measurements in the greenhouse and weighings in the laboratory.

REFERENCES.

- (1) LIPMAN, J. G. *J. Agric. Sci.* (1908-10), **3**, 297.
- (2) — *New Jersey State Agric. Exp. Sta. Bull.* (1912), No. 253.
- (3) REMY, TH. and VASTERS, J. *Landw. Jahrb.* (1931), **73**, 521.
- (4) THORNTON, H. G. Lucerne "Inoculation" and the factors affecting its success. *Imp. Bureau Soil Sci. Tech. Comm.* (1931), No. 20, London.
- (5) DORSEY, H. *J. Amer. Soc. Agron.* (1929), **21**, 679.
- (6) LYON, T. L. *Cornell Agric. Exp. Sta. Bull.* (N.Y.) (1925), No. 447.
- (7) STALLINGS, J. H. *Soil Sci.* (1926), **21**, 253.
- (8) JOSHI, N. V. *Mem. Dept. Agric. India, Bact. Ser.* (1920), **1**, No. 9, p. 247.

- (9) VIRTANEN, A. I. Über die Stickstoffernährung der Pflanzen. *Ann. Acad. Sci. Fenn. Ser. A* (1933), **36**, No. 12 (German). Summary in *Nature* (1933), **131**, 534.
- (10) ——— Undersøgelser over Bælplantebakterierne og Bælplanterne. (Danish.) *Beret. om Nordiske Jordbrugsforskeres Forenings*, IV Kongres, Helsingfors, 1929: *Nordisk Jordbrugsforskning* (København, 1929), **11**, 782.
- (11) VIRTANEN, A. I. and VON HAUSEN, S. The capability of grass plants to take advantage of nitrogen fixed by the nodule bacteria of leguminous plants. (Finnish, with English summary.) *Contrib. Lab. Valio* (Helsinki, 1930).
- (12) ——— ——— *Z. Pflanz. Düng.* (1931), A, **21**, 57.
- (13) VIRTANEN, A. I., VON HAUSEN, S. and KARSTRÖM, H. *Biochem. Z.* (1933), **258**, 106.
- (14) VIRTANEN, A. I. and SAASTAMOINEN, S. Über die Stickstoffbindung bei Erlen (Alnus). *Acta Chem. Fennica* (1933), B, **6** (German).
- (15) WARTIOVAARA, U. *Z. Pflanz. Düng.* (1933), A, **31**, 253.
- (16) NICOL, HUGH. The derivation of the nitrogen of crop plants, with special reference to associated growth. *Biol. Rev.* (In the press: to appear in Oct. 1934.)

(Received January 15th, 1934.)

THE DERIVATION OF THE NITROGEN OF CROP PLANTS, WITH SPECIAL REFERENCE TO ASSOCIATED GROWTH

By HUGH NICOL.

(Department of Bacteriology, Rothamsted Experimental Station.)

(Received February 2, 1934.)

(With Three Text-figures.)

CONTENTS.

	PAGE
I. Decline and desuetude of unitary theories of plant nutrition	383
(1) Historical and general	383
(2) The development of modern views on the nitrogenous nutrition of plants	384
II. Evidence for the utilisation by non-leguminous plants, of substances originating from the activity of nodule-bearing plants	388
(1) Pot experiments	388
(a) Uptake of nitrogen	388
(b) Uptake of phosphorus	395
(2) Accessory factors and the growth of plants; a suggested rôle for leguminous plants	395
(3) Associated growth in practice	397
(a) "Competition" between legumes and non-legumes	397
(b) Cover crops. The value of leguminous crops in a rotation	401
III. Tables summarising experiments on associated growth	403
IV. Conclusion	405
V. Summary	406
References	407

I. DECLINE AND DESUETUDE OF UNITARY THEORIES OF PLANT NUTRITION.

(1) HISTORICAL AND GENERAL.

EARLY investigators searched for an active "principle" in the soil to account for plant growth. Glauber (1656) concluded that this principle was saltpetre (cf. Nicol, 1932)—a view supported by experiments by Mayow (1674), whose findings bear a curious resemblance to the conclusions of some modern work on the course of the seasonal production of nitrates in field soils. Külbel (1741), however, looked to a *magma unguinosum* obtainable from humus as the source of fertility. Wallerius (1761) concluded that humus, being *homogeneous*, is the source of the food of plants,

their *nutritiva*, while the other soil constituents are *instrumentalia*, dissolving and attenuating the food mixture proper so as to fit it for being taken up by the plant.

Nevertheless, a defect of many early theories was that they were purely monistic: they postulated some exclusive principle. It was not until the phlogistic period that a plurality of sources of soil fertility was recognised. According to modern views, the soil, potentially a reservoir of plant food, is at any given moment functioning chiefly in relation to the plant as a support, as a substrate for the reception of added nutrients such as manures, and as an ingenious mechanism for the transport and interchange of existing nutrients carried by the soil atmosphere and by the soil water supply. (For a review of the theories of plant nutrition see Russell, 1932.) These nutrients, though centred around the substances symbolically represented by that magic trinity of letters N-P-K, now seem likely, as regards the number of elements involved, to be limited only by the range of atomic numbers. Yet, forms in which the chief known elements are considered to be available to plants are limited in number. For the majority of compounds it could be said that the simpler they are and the more familiar they were to students in the elementary stages of inorganic chemistry, the more likely have they been held to be highly available to plants. Thus, nitrogen would be taken up by plants as nitrate, more rarely as ammonia; phosphorus as phosphate; potassium as chloride or sulphate. Indeed, some of the latest developments in the manufacture of fertilisers have resulted in mere permutations of these radicles to give an ammonium phosphate intermixed with some equally simple potassium salt.

There are exceptions to the simplicity: in the case of basic slag it seems that neither can its calcium be regarded simply as "lime" nor are the phosphorus compounds available as simple phosphates. Some of the complex chemistry of the compound phosphates may have to be invoked before an understanding can be reached of the utility to plants of the phosphorus compounds in basic slag. Study of the uptake of potassium and sodium compounds seems as yet uncomplicated; problems of rock weathering and soil decomposition, at least as far as the alkali-metal nutrition of plants is concerned, appear to reduce themselves ultimately to the simplest ionic terms. Can we say the same for the nitrogenous nutrition of plants?

(2) THE DEVELOPMENT OF MODERN VIEWS ON THE NITROGENOUS NUTRITION OF PLANTS.

Simple water-soluble compounds of nitrogen are few; they consist of the series of oxidised acids and anhydrides belonging to the nitrates, nitrites, and hyponitrites; hydrides such as ammonia, hydrazine, hydrazoic acid; and intermediately oxidised bodies such as hydroxylamine and urea. The study of more complicated bodies than these would take us into deep waters of organic chemistry. It has been a very general assumption that all these substances—including simple as well as complex organic compounds of nitrogen—that are not actually toxic to plants, would if they occurred under natural conditions of field and forest be broken down, and lastly oxidised, to the form of nitrate. According to this view, nitrate is the "elemental"

form of food nitrogen for the ordinary plant: that is, the only simple form which it would encounter and the only harmless compound of nitrogen which it would take up. Some reservations were always made; thus, leguminous and certain other plants can utilise combined nitrogen resulting as a product of the activity of the nitrogen-fixing bacteria associated with the nodules, but few writers admitted the possibility of non-leguminous plants taking up compounds of nitrogen that had not undergone a change into nitrate.

Is this unitary creed too simple? At one time Liebig taught that the nitrogenous requirements of all plants were satisfied by ammonia (as such). In the light of our present knowledge there is no doubt that Liebig's "ammonia" was no more than an intelligent guess. A belief in the superior value of ammonia to plants was held for some time in France, as a result of observation by the early agricultural chemists. Boussingault was so much impressed with the effects of guano and of rotting manure that he regarded ammonia as the essential form of nitrogen. "Kuhlmann (1847) even went so far as to suggest that nitrate needed to be reduced by rotting organic matter before its nitrogen became available to the plant" (Crowther, 1934).

Liebig was wrong in being too sweeping, but it is reasonable to ask whether some of our present-day teachings concerning the nutrition of plants do not err through excessive simplification. Justification for the view that nitrate is in nature the chief, if not the only, nitrogenous building material of plants has been derived from a consideration of the nitrogen content of agricultural soils, in tropical as well as temperate climates. In such soils the content of nitrogen as nitrate, though very variable, may rise to 40 or more parts per million of soil. These amounts seem small, but it should be remembered that only about 15 per cent. of the soil is water, so that the nitrate nitrogen in the soil water, considered as a nutrient solution, is present in concentrations up to about 300 parts per million of water. To represent a concentration of calcium, sodium, or potassium nitrate these figures must again be multiplied by six or seven. Ammonia, on the other hand, is usually present in much smaller amounts in arable soils.

Until quite recently work on the soil's content of "mineral" (inorganic) nitrogen had been carried out on arable soil, frequently on fallow soil, and in any case sampling was performed in interspaces—the immediate neighbourhood of mature plant roots has been avoided. Hence it will be appreciated that the soil's content of nitrate was evaluated mainly in the absence of the plant. Reuszer (1931) found that the nitrate content of a grassland soil was minute and nearly constant. After this same soil had been ploughed up, its nitrate content increased greatly, showing, however, seasonal fluctuations. If plant roots take up ammonia, the fact would hardly have been detected by the usual methods of sampling arable land; in places bare of roots ammonia would have every opportunity of undergoing microbiological conversion to nitrate. Even a much fuller knowledge than we now possess of the soil nitrate "gradient" in the neighbourhood of plant roots would not assist us to determine whether the formation of nitrate is primarily of value to plants, or whether it represents a stage reached by nitrogenous compounds in the absence of living roots.

Other nitrogen compounds, such as proteins and their decomposition products, have been assumed to require breaking down by simplifying processes such as ammonification and nitrification; and literature of these processes forms an impressive part of the literature of soil biology and soil chemistry. Soil chemistry had its origin in Germany, France, and England, and spread to include investigations elsewhere in the temperate zone and in the tropics. It was therefore natural that findings relating to agricultural soils of these well-investigated regions should be taken as typical of most types of land, and that plants should be assumed to nourish themselves upon the nitrate which was thus recorded to be widespread and predominant in the nitrogen make-up of soils. In Sweden, however, Hesselman (1925-6) showed that in certain types of forest soils, composed largely of humus layers arising from dead leaves, the principal nitrogenous end-product of the soil biochemical processes was ammonia; further, the vegetation peculiar to these soils was well able to utilise this ammonia for growth without nitrification being necessary. In fact, some of these forest soils were almost incapable of turning ammonia into nitrate.

Hesselman is a worker who adopts a method of research that may for convenience be called the "naturalistic," or better, the "realistic." As far as possible he studies the soil just as it is, without adding anything to it or using it to grow plants under greenhouse or other artificial conditions. The natural ecology of the soil is for him the best plant indicator. Very different principles are followed by the workers who add and subtract substances to soil or to culture solutions in an attempt to elicit the response of a plant, the choice of which is often arbitrary, based upon the convenience of the worker, or upon some supposed typicality in the plant. This second method of attack has been very extensively employed; in this connection it will suffice to mention the work of Prianishnikov. In a series of papers, some of them difficultly accessible outside Russia, Prianishnikov has claimed that ammonia is efficiently utilised by certain common agricultural plants (*e.g.* peas and beetroot). Prianishnikov (1933) has written the first part of a summary of his own work. For discussion of the physiological aspects of vegetable nutrition see Crowther (1934), Onslow (1931), and Robinson (1929). Evidence is conflicting as to whether ammonia or nitrate is the more efficiently utilised by plants, but there is considerable agreement that they can use either source.

The overwhelming importance that has been attributed to nitrate would seem to have arisen largely from the unequal extent to which arable land, grassland, and forest soils have been investigated. If we admit that ammonia, as well as amino- and amido-nitrogen, is important, not merely in the nutrition of the flora of particular areas of forest but also throughout the wide expanses of grassland and prairie, the nitrate castle crumbles and we are no longer able to defend the position that there is a unique source of nitrogen for non-leguminous crops.

Grassland offers us an opportunity for studying the uptake of nitrogen under conditions such that all the available soil is penetrated with plant roots. Hall, Miller, and Gimingham (1908) were among the first to break away from a pre-occupation with nitrate as the principal source of nitrogen for plants. They

suggested that grass in acid soils "must, in the main, utilise the ammonium salts without previous change." Surprisingly little work has been done since 1908 on the mineral nitrogen relationships of grassland. Richardson's (1933) work on the fate of additions of ammonium salts to Rothamsted grass soils of various reactions has confirmed this supposition that grassland vegetation can directly assimilate ammonia.

The weight of observational evidence in the ordinary arable field has been supposed to support the view that nitrogenous manures must be supplied in the form of nitrate or must be converted to nitrate before being available to agricultural crops (cf. Beaumont and Moore (1933) for a summary). It is common knowledge that if undecomposed or partly decomposed nitrogenous manure, not containing much nitrate, is added to soil just before planting a crop, the crop usually suffers therefrom. A counsel of perfection is to apply farmyard manure always to the previous crop—jam yesterday but never jam to-day. Farmyard manures and the like contain comparatively large amounts of the simpler organic compounds of nitrogen, derived from decomposition of protein. Some at least of these simpler substances should be economically available to plants, if Prianishnikov, Mevius, Mothes, and others are right. Yet age-long practice counsels against the application of fresh dung. The mere absence of nitrates might account for a starved plant, but could not harm the plant to the extent commonly anticipated.

Two explanations are available for the harmful effect of fresh nitrogenous manure. One takes account of the carbohydrate material—usually straw or other plant residues—which is added with most of the common farmyard manures or simply as "green manure." According to this theory, when carbohydrate material (with the nitrogen compounds contained in the plant residues as well as that derived from urine and faeces) is decomposed, swarms of fungi and bacteria pullulate upon the carbohydrates. Kalatshikov (1928–30) has shown that in three Russian soils, in presence of pure starch, the nitrogen of ammonium sulphate and of calcium nitrate can be largely taken up in the tissues of micro-organisms (cf. Murray, 1921). They "lock up" not only nitrogen from the manure but also the soil's own nitrogen which is thus rendered unavailable to plants, until such time as the micro-organisms themselves decompose (Jensen, 1932; Doryland, 1916). This explanation is undoubtedly well founded as far as it relates to strawy dung and new composts, and to the repressive effect of ploughing-in undecomposed carbohydrate materials such as fresh straw. But it fails to account for the behaviour of amendments not notably rich in carbohydrates, such as human night soil and poultry manure, about which the expression "hot" is often used. Some other explanation must be sought, and hitherto the absence of sufficient amounts of nitrate has been taken—somewhat inadequately—to account for the nutritive aspect of the question.

Barritt (1933*a*) has put forward a theory based on numerous experiments, according to which the repressive effect of fresh organic nitrogenous material on the growth of nitrifying bacteria is due to the accumulation of carbon dioxide and ammonia, resulting from the activity of micro-organisms. The soil atmosphere is

vitiated and the partial pressure of oxygen is reduced. According to Barritt, an unfavourable result from this unnatural atmosphere is a suppression of the formation of nitrates by the nitrifying bacteria. Winogradsky claimed that nitrifying bacteria could not function in presence of organic matter. Barritt takes a much less extreme view of the restrictions of their biotic requirements. The force of Winogradsky's claim has been considerably reduced since the discovery of ammonia-oxidising bacteria capable of living on organic media (Cutler and Mukerji, 1931; Cutler and Crump, 1933); indeed, it would seem absurd to credit the nitrifying bacteria with any activity in soil if they are as exacting as Winogradsky claimed. Barritt's view accords well with what one would expect to find in soil: he has successfully resolved a number of apparent contradictions respecting the natural course of nitrification.

Barritt's theory thus brings us back to the question of the value of ultimate nitrification. He thinks that nitrification is desirable, but he did not consider, in that paper (1933*a*), the question of how conditions will affect the *plant* when so much carbon dioxide and ammonia has accumulated as to stop nitrification. It seems highly probable, nevertheless, that a poorly oxygenated soil atmosphere and soil solution, rich in carbon dioxide and free ammonia, would be unfavourable to plant growth on account of the effects upon root respiration. Willis and Rankin (1930) have demonstrated injury to plant roots arising from liberation of free ammonia when cotton-seed cake was used as fertiliser. In agreement with Barritt, it was found that such excessive ammonia could not be nitrified. Jashnova (1930), however, found that nitrification of high amounts of ammonia was delayed only if phosphates were present in insufficient quantity. It is understandable that a partly asphyxiated plant would be unable to profit from any nitrogenous constituent of its environment. On this view, the plant cannot profit from organic manures while their organic matter is in a state of rapid decomposition. Thus, a high degree of nitrification may imply little more than that those decomposition processes which pollute the soil atmosphere have reached a term. The plant may, however, use nitrate as a source of oxygen (Barritt, 1933*b*). The formation of nitrate from rapidly decomposable organic nitrogenous manures would, on this view, be an index of substantial cessation of the processes of decomposition. It would not necessarily be an end desirable in itself.

II. EVIDENCE FOR THE UTILISATION BY NON-LEGUMINOUS PLANTS, OF SUBSTANCES ORIGINATING FROM THE ACTIVITY OF NODULE-BEARING PLANTS.

(I) POT EXPERIMENTS.

(a) *Uptake of nitrogen.*

The manurial use of complex protein material such as dung, horn, feathers, carrion, and so on is of high antiquity, but owing to the preoccupation of soil chemists with the "nitrate theory," very little exact work has been done on the

parts taken by organic nitrogen compounds. Latterly, simple nitrogen compounds of carbon—urea, cyanamide—have assumed commercial importance, but their fertilising function in soil has been assumed to be related to the effectiveness and rate of their ammonification, with subsequent nitrification. In the complex biodynamical equilibrium of soil, it may be true to say that urea and cyanamide are ultimately nitrified, as are all other nitrogen compounds, when not taken up, at some stage or other of their decomposition, by the plant. But just because the soil is so complex and biologically active it is difficult, if not impossible, to determine exactly what happens within it to a decomposable addition.

Recourse must therefore be had to the simpler conditions offered by sand or liquid cultures in small vessels. A review of most of the early work with single plant species was made by Hutchinson and Miller (1911). In their original investigations these authors showed that plants (peas and wheat, separately grown) can absorb some water-soluble organic nitrogenous compounds.

Suggestive results have been obtained from experiments on the growth of two or more species simultaneously in one culture flask or pot. Especially valuable have been the experiments on the associated growth of leguminous with non-leguminous plants. Agriculturalists rarely aim at growing two or more non-leguminous species together, unless one or more species of legumes be present. The culture of "Maslin," "mashlum"—mixed cereals—is an exception, as is the growing of a weedy crop. Sometimes two or more grasses are grown in mixture, but much more frequently one or more clovers or other legumes are incorporated in a mixture of grasses. Forage crops, which, like the yield of grassland, are intended solely for consumption by animals on the farm, and not for sale at market, also commonly comprise several species. When they do, at least one legume is an almost invariable component. (Intercropping in market gardening on highly manured land is a distinct kind of operation which need not be considered here.) It is, moreover, a common practice to under-sow legumes in a cover crop of cereals (see section "Cover Crops," p. 401).

Thus practices have developed which suggest that leguminous plants have a singular rôle in agricultural economy. The Roman writer, Varro, remarked upon that property of leguminous crops which we should nowadays call restorative: that is, he noted that a legume crop increased the fertility of soil and benefited a succeeding non-legume. This valuable property of legumes was systematically utilised in the process of rotation of crops, whereby once in a period of years—usually every fourth year—clover or beans were sown. It was not until 1886 that the outstanding peculiarity of leguminous plants was discovered to be due to their ability to capture atmospheric nitrogen by the aid of bacteria living within the root-nodules. Somehow, by a process not yet understood, the joint action of nodule bacteria and plant tissue made available to the leguminous plant that atmospheric nitrogen which was unavailable to all high animals and to nearly all other higher plants. Even in a poor soil, the legume thus became rich in nitrogen—richer than most non-legumes—and, dying, left a nitrogen-rich root residue which decomposed and acted as a source of available nutrients to its successors.

This property of legume roots of increasing the soil's fertility by their subsequent decomposition has no obvious bearing upon the question of associated growth in conditions where the beneficial effects of the legume often occur before much root decomposition can have occurred. Only during the present century have conscious attempts been made to relate the two things.

It is not overstating a case to say that a mixed vegetation is symbiotic. Leather (1897) seems to have been the first to appreciate the possibility of this being so. He wrote: "The question naturally arises, are the Papilionaceæ able to assist in any way the plant of another natural order *which is growing alongside them.*" Observations of the Indian practice of growing gram (*Cicer arietinum*) and wheat together, led to this speculation. Farmers have often remarked on enhanced vigour in non-legumes growing with legumes, and it would appear that science once again has lagged behind practice. It was from consideration of field growth of mixtures of Canada peas and oats that J. G. Lipman was led to make in 1908 what appear to be the first pot experiments to have a bearing upon the question of transfer of nutrients in associated growth. Lipman claimed (1912) that his interest in the subject arose prior to 1905, but the first (albeit an anonymous) publication that can be ascribed to him appeared in 1910*a* and related to experiments made in 1909. It was not until 1912 that he published the results of the experiments made in 1908, when, possibly, he had not the courage of his convictions. Lipman's work included an examination of the effects both of withholding and of adding artificial nitrogen to the mixed vegetation; his experiments remain the only ones expressly designed to examine the effect of added nitrogen upon the uptake by graminaceous plants of nitrogenous substances derived from host legumes.

Lipman's first published (1910*a, b*) experiments were only qualitative, but they resulted in "a striking proof of the ability of oats to secure an adequate supply of nitrogen when growing together with field peas in a soil devoid of nitrogen" (1910*b*). The method used was the simple one of growing the two plants in sand; the species were separated by either a porous or a non-porous pot. When separated from the peas by a porous partition, oats grew better and became much greener than the oats in a glazed and presumably impermeable pot. Since the effects were marked before the plants were ten weeks old, nitrogen derived from root decomposition would seem to be practically excluded from consideration. Lipman worked with numerous other mixtures, both in pots and in the field. It is curious to note how all the schools of subsequent workers upon associated growth have been unaware of previous work at the time of commencing their own. Lipman's work having escaped their notice, Lyon and Bizzell published in 1911 a paper entitled "A heretofore unnoticed benefit from the growth of legumes." Lyon and Bizzell's field experiments upon which their paper was based led to the same broad conclusion as Lipman had already drawn. Lyon and Bizzell's analyses showed that in the field a non-legume could attain a higher protein content when grown with a legume than when grown alone. (For a polemic regarding the question of priority, see Lyon and Bizzell (1913*b, c*) and Lipman (1913).) Lyon and Bizzell concluded that the increased protein content of a graminaceous plant grown in

association with a legume was due to nitrification promoted by the presence of the legume. Another paper by the same authors (1913*a*) discusses the query whether there is a mutual stimulation of plants through root influence. Though a number of field experiments on mixed vegetation of grassland were performed (good examples are the work of Evans (1916) and of Skinner and Noll (1919) (nitrogenous manuring)) no further work on the mode of uptake of nitrogen by one plant from another can be traced until 1926, when Stallings published a paper on "The form of legume nitrogen assimilated by non-legumes when grown in association." Stallings wrote: "That non-legumes, when grown in association with inoculated legumes under favourable conditions, profit by the association is a well-established fact," but, in his paper no bibliographical reference to the question was given. He worked with the curiously unpractical association of wheat and soya beans, grown separately as well as together. Twenty-four tables laboriously give a picture of his analytical results. The recorded crop weights and nitrogen contents suggest that the crop and absolute nitrogen yields were depressed or at best not increased, by associating the two plants, but this appearance may be due to the involved method of presentation. However, Stallings was not concerned with the crop yields, but with elucidating the nature of the substance presumed to have been transferred from soya to wheat. He concluded that "the beneficial influence exerted upon wheat by the inoculated soybeans was evidently due to soluble nitrogen, possibly ammonia, placed at the disposal of the latter by the former, when grown in association." Stallings's conclusions were vague, but he deserved credit for being the first to attempt to determine the form of nitrogenous compound excreted and taken up.

Comparing the nitrogen nutrition of red clover and white clover supplied with effective nodule bacteria, and with that of the same species supplied with ammonium nitrate under sterile conditions, Virtanen (1928, 1929) concluded that these species differed in their ability to make use of the nitrogenous compounds originating in the nodules. He was thus led to try with von Hausen (1931) the effect of various amino-acids, as well as inorganic compounds of nitrogen, as sole source of nitrogen for these plants. In 1927 Virtanen and von Hausen (1931) had noticed that when inoculated red clover was grown alone in sand, notable amounts of nitrogenous material were to be found in the sand. An excretion began at a very early stage of growth and was also observed to take place with vetches in water culture (Virtanen and von Hausen, 1931). These ingenious researches needed but three steps to complete them, namely, to show that non-legumes growing alone were able to utilise amino-acids; that amino-acids were excreted by leguminous plants, and that non-legumes could profitably utilise those excretions of legumes. All of these steps were made.

Virtanen's first experiment on associated growth was performed in 1927 and reported in 1928 and 1929. It consisted simply of a demonstration that in absence of added nitrogen, oats grew sturdily when associated in a sand culture with inoculated peas, but failed to make any growth when the peas were not inoculated.

The next experiments by Virtanen and von Hausen (1930, 1931) were made upon red clover and meadow foxtail grown in association. These authors were the

first to introduce the notion of an effect of the proportion of non-legumes to legumes, though Lipman had already (1912) thought it worth while to record the numbers of each. These experiments with clover and meadow foxtail were performed in the successive years 1928 and 1929 with the species in the ratio of 1 and 2 grass plants for each leguminous plant. Both species and both sets of experiments showed a curiously wide variation in yields in the two years. No parallel pot and no control was set up. The experiments were made with sets of four pots, each pot being maintained at pH 5.0, 5.5, 6.0 or 6.5. This examination of the effect of varying degrees of acidity was another new feature of the investigations of associated growth. It was a logical consequence of earlier work upon varying the pH of growth of cultures of isolated bacteria; this in turn led to a comparison of the results of growing single species of leguminous plants: (a) in conjunction with their own nodule bacteria, and (b) aseptically fed with mineral nitrogen. The pH that was optimal for one species of the vegetation would not in general be optimal for another species. The maintenance of a range of acidities (cf. Wood, 1933) was desirable for a thorough examination of the effectiveness of any transfer of nutrients—if indeed, transfer be the word for so passive an uptake.

In addition to the red clover-meadow foxtail mixture, Virtanen and von Hausen (1930, 1931) investigated the growth of mixtures of peas and oats in the ratio 1, 2, 2.75, 4 and 5 oat plants per pea. These proportions were grown in pots at pH 6.5, which was optimal for the peas and also for the variety of oats (*Argus-Hafer*) employed. A further experiment with oats and peas grown in equal numbers at pH 5.5, which is unfavourable to the legume, but well tolerated by oats, showed that at that acidity the oats could still obtain considerable quantities of nitrogen from the peas. The authors also grew white clover with cocksfoot, and a composite mixture consisting of red, white and alsike clovers and timothy, meadow foxtail, cocksfoot, and *Poa* sp.

In all the Finnish experiments so far described, except in the red clover-meadow foxtail mixtures subjected to adverse conditions, favourable growth of the graminaceous constituents of the mixture was observed. The growth of the non-legumes was in fact of the same vigour as if an adequate supply of nitrogenous manure had been given. Nevertheless, there was evidence that when the ratios of the numbers of non-legume plants to leguminous plants approached or exceeded 2 : 1, the growth of both species suffered; in the case of five mixtures of peas and oats the set-back to growth was even more evident in the peas when the cereal was numerically preponderant.

The remaining pot and field experiments by Virtanen and his school, upon the question of associated growth, confirm the findings already given, and need little comment (see Table II below). General conclusions have been summarised by Virtanen (1933 *b*), Vartiovaara (1933), and in *Nature* (Anon. 1933). Mention must be made of the work of Virtanen (1933 *b*) on growing plants individually with their roots in sterile "closed systems" (plugged flasks containing sand and nutrients). As stated above he was able to show that under such conditions, red clover (*Trifolium pratense*) profitably utilised aspartic acid and the products of hydrolytic cleavage of

casein, whereas white clover (*T. repens*) was better nourished from ammonium nitrate. Inoculated peas excreted nitrogen in the following proportions (Virtanen, von Hausen and Karström, 1933):

Nitrogen as	% of total nitrogen
Amino-N	77.4
Ammonia-N	0
Amide-N	3.30
Volatile basic N	2.73
Melanin (humus) N	2.05

No results have been published concerning the ability of oats in single culture to take up amino-compounds. Peas, barley and wheat made use to varying extents of hydrolysed casein and of six single compounds of mineral and amino-acid nitrogen. Aspartic acid, and asparagine to a less marked degree, were claimed to be suitable for the nitrogenous nutrition of leguminous plants, at least during the first 5-7 weeks of life. Barley and wheat better utilised the nitrogen of potassium nitrate during a similar period. Such experiments upon the nitrogen nutrition of single species are not new in conception (*e.g.* Nakamura, 1894-7): these results are recorded here solely on account of their relation to the problem of associated growth. Evidently, more work requires to be done upon the question of the forms of nitrogen metabolised and excreted by legumes and assimilated by non-legumes grown in association with them. Virtanen (1933 *b*) has suggested that plants can obtain part of their anabolic carbon from nitrogenous compounds of that element.

Virtanen, von Hausen and Karström (1933) grew one leguminous and one oat plant together in flasks maintained under conditions sterile except for the presence of specific legume nodule bacteria. The plants were sometimes entirely within the glass vessel. An interesting variation of this technique was the growth of peas and oats in three-necked Woulff's bottles; each aseptic bottle contained the roots of one inoculated pea plant and one oat plant growing in sand, while the aerial part of each plant emerged through one of the necks. This latter arrangement permitted a freer development of the plants. In all such experiments, the inoculated legume successfully acted as nitrogen foster-mother to the oat.

The alder, though not a legume, bears upon its roots nodules produced by nitrogen-fixing bacteria. Virtanen and Saastamoinen (1933) have shown that inoculated alder plants excrete nitrogenous substances into sand in a pot culture, whereas an uninoculated plant did not excrete an appreciable amount of nitrogen. Inoculated alders made better growth than alders not inoculated but supplied with ammonium nitrate as sole source of nitrogen.

The reality of the production of nitrogen compounds by the roots of one plant, in forms in which they are available for the nutrition of another plant, can now be considered as established, though the mode of excretion is still obscure and many possible factors have not yet been tested. In 1931 Thornton and Nicol (1934) set up a pot experiment in sand, intending to examine some aspects of nitrogenous manuring upon competition between a legume and a non-legume. Nitrogen as

sodium nitrate was added at three levels of manuring but in one dose to each of a number of parallel pots, to which basal nutrients were also given. Lucerne (var. Grimm: *Medicago sativa* \times *M. falcata*) and Italian rye grass were then sown and the seedlings were thinned out to leave equal numbers of plants of each species. Lucerne was also grown alone under the same conditions. The lucerne seed was inoculated with an efficient strain of lucerne nodule bacteria. Three parallel pots of each treatment were removed when the plants were 2, 3, 4 and 5 months old, and the plants were harvested so as to secure both roots and tops of each kind separately. These successive reapings enabled the course of growth to be represented graphically, and in this respect this experiment is unique among experiments on

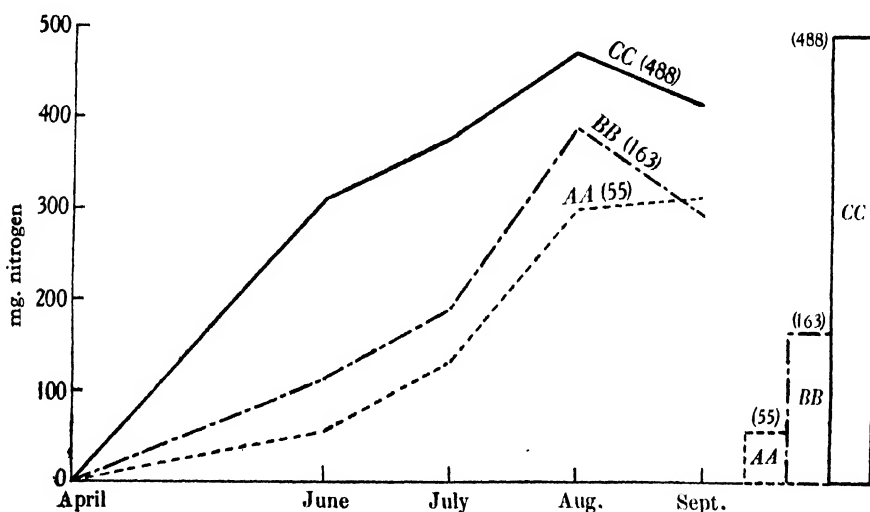


Fig. 1. Milligrams of nitrogen in tops and roots together in Italian rye grass grown with lucerne at three levels of nitrogenous manuring. The columns at the side represent on the same vertical scale the amounts of nitrogen (as sodium nitrate) added in one dose at the commencement of the experiment.

associated growth. In the pots that had received the two lowest dressings of sodium nitrate, the grass had a content of nitrogen equal to several times the amount supplied in the manure. Fig. 1 shows the nitrogen contents in the grass (whole plants). The content of nitrogen in the grass growing in those pots that received the largest dressing of sodium nitrate closely approached a figure which might have suggested 100 per cent. recovery of the added nitrogen. It is nevertheless impossible to say how much of the grass nitrogen was derived from the sodium nitrate, and how much from the lucerne. It is evident that when growth of the lucerne was not depressed by nitrogenous manuring, the grass contained nitrogen which could have come only from the legume. Even in the most highly manured series, the grass had probably derived some nitrogen from the lucerne.

(b) *Uptake of phosphorus.*

Though numerous experiments on root excretions have been made, only one has been found with a bearing upon the subject of associated growth. Domontovich, Shestakov, and Polossin (1933) found that oats in association with lupins took up much more phosphorus from phosphate rock than when grown alone. In this association the effect upon the non-legume of the legume's root excretions was indirect; it was due to the lupins dissolving more phosphate than they required. It does not appear to have been determined whether nitrogenous compounds are concerned in the solubilising effects. Probably other examples of this type of association will be revealed; cf. Prianišnikov (1934). T'yagny-Ryadno (1933) has described such a solvent action upon phosphorite, due to *B. mycoides*.

(2) ACCESSORY FACTORS AND THE GROWTH OF PLANTS; A SUGGESTED
RÔLE FOR LEGUMINOUS PLANTS.

Many agriculturists are beginning to think that accessory factors are somehow concerned in the nutrition of plants. The accessory substances (vitamins, auximones, growth and co-growth substances, phytamins) are not necessarily compounds of nitrogen, but the question of their production is relevant to problems of manuring and will be briefly discussed. There are several aspects which it is essential to distinguish. These are summarised below.

Sources of possible accessory factors in plants, whether required for the proper growth of the plants themselves or for animals which ingest them.

- (1) Derived from inorganic compounds of nitrogen:
 - (a) Directly built up by and within the plant.
 - (b) Synthesised by micro-organisms living free in the soil.
- (2) Derived from organic sources of nitrogen:
 - (a) Synthesised by nodule bacteria:
 - (α) In symbiosis with their host plant and used wholly within that plant.
 - (β) In symbiosis with their host plant, but taken up by associated plants.
 - (b) Found in organic manures derived from land or marine plants or animals:
 - (α) Already formed therein (i) in organs and tissues, (ii) by biological activity in the gut of animals.
 - (β) Formed by the action of micro-organisms (including free-living nitrogen-fixing organisms) thereupon.

Hitherto, experimental investigation has been almost exclusively devoted to the vitamins (required by animals) produced in germinating and mature plants. The production of vitamins in plants in cultures has been extensively investigated—by the school of Virtanen, amongst others. It should be borne in mind that vitamins, however essential for animal growth, may be a waste product in plants. Virtanen (1933 (b)) was tempted to conclude that the production of vitamin C and of the pro-

vitamin, carotin, was correlated with the growth of the plant, and that these substances are growth factors of plants. This tentative conclusion would suggest that the plants investigated were "raising themselves by their own boot-straps." It is reasonable to suppose that the vitamins needed by the animal may not be identical with the accessory substances required for the plant growth. It is therefore convenient to distinguish accessory plant-growth substances as phytamins. Evidence is accumulating respecting their reality.

Viswa Nath (1932) and his co-workers at Coimbatore and elsewhere have taken a prominent part in investigating the influence of manuring upon vitality of plants (Viswa Nath and Suryanarayana, 1927). Indian cattle manure had a high value in this respect, but the unsaponifiable matter of cod-liver oil, as well as farmyard manure extract, stimulated growth and reproduction of plants. Hartley and Greenwood (1933) in Nigeria, and Tyagny-Ryadno (1933) in Russia, have recorded effects from small amounts of farmyard manure greater than could be accounted for from consideration of its content of common nutrient elements alone. The extent to which vitamins are produced by rational nitrogenous manuring of crops is of sociological importance and deserves attention (Armstrong, 1933). This aspect of crop manuring has a bearing upon the ability of animals to resist disease (McCarrison, 1926; Howard, 1933). Hartelius (1933) has reviewed the literature on the "growth substance B" that occurs in urine and stimulates the growth of plants. Extracts of root, stem and growing tip, of seedlings of maize three or four days old were found by Popoff (1933) to stimulate the growth of *Euglena gracilis*; this may have been due to vitamins, in which young plants are rich, or to amino-acids. Thornton and Smith (1914) noted a marked stimulation of *Euglena* by presence of amino-acids, especially tyrosine in aqueous solution. Mockeridge (1920) thought that true plant-growth accessory substances were nucleic acids or their constituent nitrogenous bases. An extensive field of work appears to be open.

It is not known to what degree plants rely upon animal excretory products. Results from Agdell Field, Rothamsted, show that the ratio of the yield of barley on the "clover" side to that on the "fallow" side (without clover) appears to be altering. No dung has been carted on to these plots since about 1844, but, for a time, sheep were fed on the turnip crops upon the field. The last record of animals on the field was made in 1876. The suggestion that some of the crops may be suffering from an insufficiency of some substance contained in dung is supported by consideration of the "continuous clover" plot, which was laid out in richly manured garden soil in 1854. Since about 1874, increasing difficulty has been experienced in getting the clover to grow and maintain itself on this plot.

The poor quality of continuously grown cereals shows that dung alone is not enough. There remains to be ascertained how much the benefits of sequence, as well as association, of plants are referable to accessory substances (phytamins) produced by members of the association. It may well be that nodule-bearing plants have a peculiar aptitude for the production and bequeathing of phytamins. Residual effects from leguminous crops, especially lucerne (Nicol, 1933), have been observed to persist for many years. It is possible that the manurial value of legumes, as well

as of animal manures, is not entirely due to their leaving a residue of mineral nutrients.

If these notions can be shown to be well founded, new light may be thrown not merely on the problem of mixed vegetation, but also upon the value of rotation of crops in the field and upon the succession of plants in nature.

(3) ASSOCIATED GROWTH IN PRACTICE.

(a) "*Competition*" between legumes and non-legumes.

Of great practical importance to agronomy has been the recent introduction to grass mixtures of seed of wild white clover, a perennial. Clovers had long been incorporated in "seeds" (see p. 401), but the introduction of "wild white" gave such remarkable results in the improvement of grassland that it ranks as a fresh step. It had been noticed that certain Kentish pastures wherein wild white clover was abundant had an exceptionally high feeding value. Seed of the clover was saved and distributed, and now the "wild white" is cultivated—though "original Kentish" still fetches the highest prices. In other countries advances have also been made in respect of the leguminous component of herbage: e.g. in Austria (Jentsch, 1927); in Australia "subterranean clover" (*Trifolium subterraneum*) has been extensively introduced, while New Zealand produces an export surplus of wild white clover seed. In three decades or so the value of wild white clover has been so fully recognised that it may be said to be invariably a component of the best seed mixtures used for establishing permanent grass.

The period of introduction of "wild white" coincided closely with the period of development of new nitrogenous fertilisers intensively marketed. Many of them, as well as the older preparations, were used on both newly sown and on old clovery grassland, but the results obtained were often equivocal. So long as evidence of the bulk yield of crop was accepted, small progress was made concerning the real effect of nitrogenous manures upon swards, but when exact botanical and chemical investigations of the manured crop were multiplied it came to be recognised that nitrogenous manures were seldom of benefit (Brown, 1932*a, b*). In one of the earliest publications of Lawes and Gilbert (1858) the statement was made that "increased growth of the *Leguminous herbage of the meadow* was not favoured by the direct supply of nitrogenous manures," but their observations remained almost isolated until comparatively recently. Now the substance of the above-quoted remark of Lawes and Gilbert is accepted practically everywhere. Workers on the manuring of grassland in many countries have come to the same almost unanimous conclusion: when legumes are a component of a mixed vegetation, an addition of combined nitrogen is of little or no benefit in increasing the nutritive value of the crop.

The form of nitrogenous compound or compounds built up by the nodule bacteria cannot be said to be known with certainty, but it is probably an organic compound. Leguminous plants are nevertheless capable of having the nitrogen gained from their nodule bacteria superseded or replaced by externally applied

nitrogen. The nodule bacteria may be entirely lacking, under either natural or experimental conditions, or the activity and numbers of the nodules may be suppressed by the action of large doses of nitrate: in any such event, added nitrate, or ammonium salts, will enable leguminous plants to function as non-legumes, and take up inorganic nitrogen. It is not usually economic to allow bought nitrogen to supersede the natural gratuitous building up, by the nodule bacteria, of atmospheric nitrogen into plant tissue.

The benefit derived by non-legumes from nitrogenous manures is thoroughly established. It seems curious, therefore, that an addition of combined nitrogen to a mixture of the two classes of plants, each of them separately able to utilise the manure, should result in no apparent gain of harvested crop or in the number of animals fed off a given piece of land. (The still recent problem of "rotational grazing" is almost as much a matter of animal husbandry as of agronomy and deserves separate treatment; cf. Jones (1934).) This widely recorded failure of mixed vegetation treated with artificial nitrogenous manures to increase the yield of protein and of nitrogen recovered in the crop is well instanced by the results of field experiments at Rothamsted, of which two may be quoted here. A forage mixture experiment (oats or barley, with vetches or peas, and with a basal sowing of field beans) was commenced on arable land in 1930, ammonium sulphate being used as nitrogenous manure with a full basal dressing of phosphatic and potassic manures. In its first year the following results were obtained (all mixtures):

Nitrogen added (cwt. per acre)	0	0.2	0.4
Yields of dry matter (cwt. per acre)	23.3	31.8	35.8
Percentage of crude protein in crop	11.7	9.6	8.6
Nitrogen in the crop (cwt. per acre)	0.42	0.44	0.44

The bulk of the crop was increased, so that an uncritical estimate by eye, or by weight alone, would have produced testimony to the virtue of the nitrogenous manure. It can be seen at a glance, however, that no significant proportion of the applied nitrogen was recovered in the crop. A similar result was obtained in 1931, when nitrogen in the forms of ammonium sulphate and sodium nitrate were supplied to combinations of cereals and legumes. (See *Rothamsted Reports* for 1930, 1931, and 1932, and Tables II and III below.)

In 1932 another field experiment was set up, using oats and vetches only, in several proportions. Some results of this experiment are recorded diagrammatically in Fig. 2.

Differences between the different seeding rates are significant, but the total nitrogen contents of the crops were not appreciably altered by the application of nitrogen (as ammonium sulphate). The total nitrogen content was maximal with both treatments for a mixture of 50 lb. oats and 150 lb. vetches per acre, and was absolutely highest when no nitrogenous manure was applied to a mixture of that composition.

In the lucerne and grass (pot) experiment by Thornton and Nicol (1934) sodium nitrate depressed the growth of a mixture of lucerne and grass. In those

pots which had received most nitrate, the lucerne, in association with grass, grew much less strongly than in the less highly manured pots (Fig. 3). It is therefore fair to draw the inference that the more highly nitrogenously manured lucerne plants had less nitrogen to place at the disposal of the grass. The nitrate increased the growth of the grass (at least the two highest doses did) and the factor of root competition entered in. The addition of nitrogen as sodium nitrate to this particular association progressively reduced the total nitrogen content of the mixtures and was cumulatively adverse to the leguminous partner.

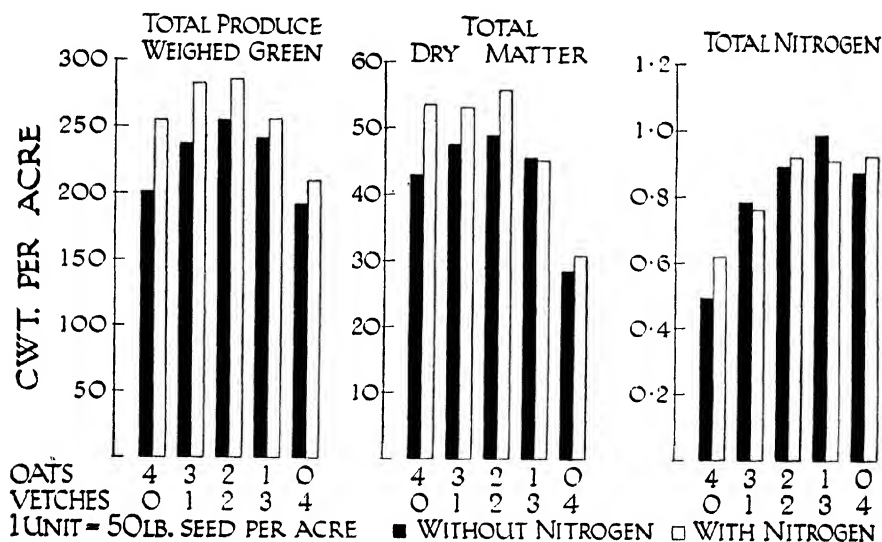


Fig. 2. Fodder mixtures of oats and vetches, Rothamsted.

On grassland the almost invariable effect of nitrogenous manures applied to mixed vegetation is to repress the nitrogen-rich legumes and assist the non-legumes. Only two explanations are apparent: either (a) combined nitrogen is toxic to leguminous plants, or (b) competition between the two sorts of plants results harmfully to the legumes when combined nitrogen is artificially supplied to the mixture.

The question of toxicity need not detain us. Ammonium sulphate has long been used to "kill out" clovers on lawns. It may be that its action is due to its strengthening the grasses, and it was also thought that ammonium sulphate, being "physiologically acid," made the soil acid to a point when the clovers languished. Blackman (1932) who worked with ammonium compounds—the sulphate, and also the phosphate, which is not so "physiologically acid" as the sulphate—found that a perceptibly toxic action of the ammonium radicle upon weeds, though not upon clovers, manifested itself before the soil had become acid.

A possible explanation of the observed facts lies in the effects of "competition"—the reaction of the members of the plant population upon one another. The extent

of the occurrence of plant reactions and coactions is dimensionally limited, but the investigation is not greatly facilitated by that fact; no mathematical treatment, for instance, can be applied. The "realist" method of studying the botanical ecology

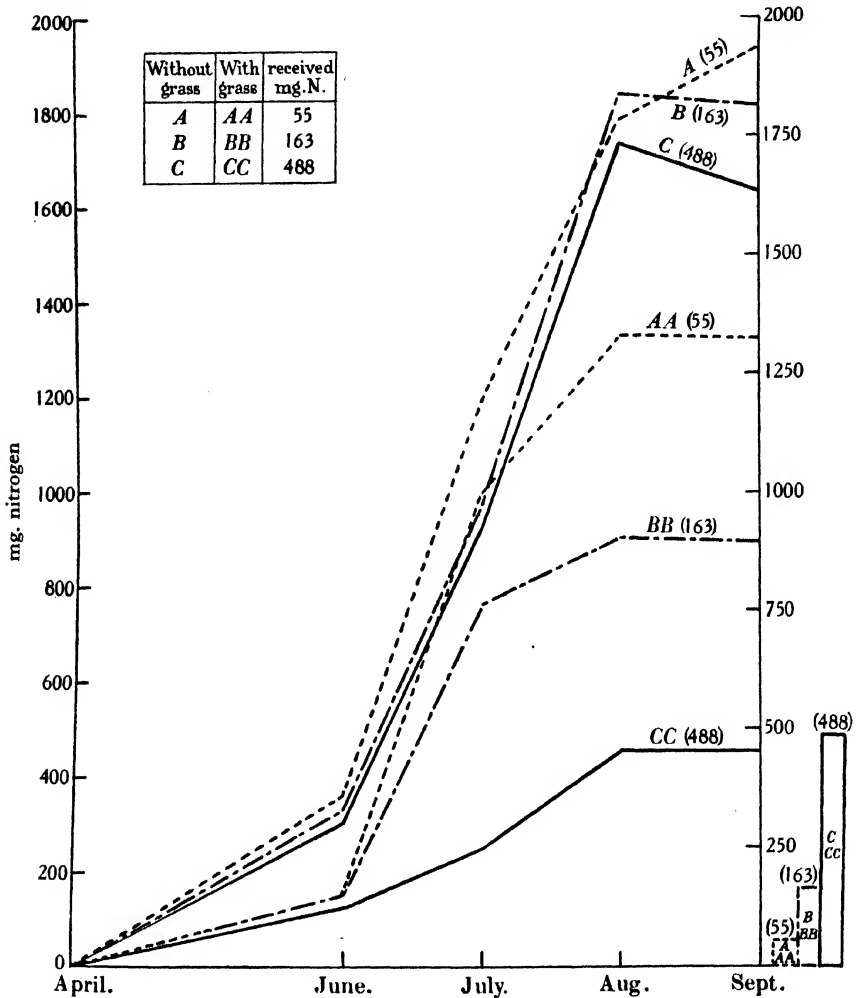


Fig. 3. Milligrams of nitrogen in tops and roots together of lucerne grown with and without Italian rye grass at three levels of nitrogenous manuring. The columns at the side represent on the same vertical scale the amounts of nitrogen (as sodium nitrate) added in one dose at the commencement of the experiment.

of mixed vegetation (at least as it occurs in grassland) has been relatively well developed since Lawes and Gilbert parcelled out the first experimental meadow in 1856, but botanical analysis even in conjunction with manurial trials tells us little about the mechanism of competition. Exact knowledge about the way one plant

acts upon its neighbours has necessarily been obtained with pot cultures, maintained sometimes under conditions of strictly controlled sterility. Such controlled and unnatural methods, it may be recalled, were the only ones of value in deciding the question whether symbiosis existed between the leguminous plants and their nodule bacteria.

Applied to the study of the problem of associated growth, both laboratory and field experiments have demonstrated that the "competition" of legumes and non-legumes can be made to assume a considerable value to man. With the help of the information furnished by such experiments, man himself can benefit from the association of these plants. Associative growth is in fact growth in equilibrium, and only when this equilibrium is destroyed does the competition factor become noteworthy.

(b) Cover crops. The value of leguminous crops in a rotation.

Sometimes the term "cover crop" is applied to an intercrop in an orchard, or to temporary and subsidiary crops intended to cover the ground before a ploughing-in as green manure. In British practice, and in the only sense in which they will be discussed here, cover or "nurse" crops consist of a single species, usually a cereal, amongst which another and usually leguminous crop is sown. Use of the term "nurse" crop for the cereal seems unfortunate, since the cereal tends to rob rather than to protect the undersown legumes.

Lipman's review (1912) of some historical references to the practice of sowing legumes in cover crops is sufficient to indicate the extensiveness of observations made thereupon. Jentsch (1927) has given an account of the little-known "Egartwirtschaft" practised over the greater part of southern Austria; this rotational system is based on undersowing with clovers.

In Britain, however, very few experiments have been made for the purpose of determining the mutual actions of a cover crop and its undersown crop. This is surprising in view of the agricultural importance of the practice of sowing clover in barley, for example. The still controversial results of the Duke of Bedford and S. U. Pickering (1919) deserve mention, but they cannot be briefly discussed.

With a cereal and a legume, the effects of competition are recognised in practical agriculture to the extent of advising that if a cover crop is used at all, the sowing of non-legumes should be thin to give the legume a good start when the legume is to be the principal crop, as in the case of lucerne sown under a cereal. Commonly the cereal is intended to be the principal crop, so that a normal sowing of the cereal is made, and the usual nitrogenous fertilisers are applied. In such cases the legumes are clovers sown either singly or as a mixture of clovers and grasses. This undersown crop (the so-called "seeds" ley) is intended for cropping in the year following the cereal, and the effects of competition upon the legumes are tolerated during the year of sowing. These effects are due to dense sowing and nitrogenous manuring of the cereal, as well as to restriction of light by the maturing cereal crop.

Undersowing a leguminous crop in a cereal differs from growing a fodder crop, inasmuch as the undersown crop is not intended to be harvested with the cereal.

Numerous field trials made by Thornton (1929*a*) showed that inoculated lucerne was better able than uninoculated to make good growth under a cereal cover crop even where some wild nodule bacteria were present. Supplying active nodule bacteria by "inoculating" the seed gave the lucerne a good start, and this produced a lasting effect upon the growth of the lucerne. Since the only comparisons made were between lucerne inoculated and uninoculated, sown bare and under a cereal, no crop yields of the cereals are available. Agdell Field four-course rotation, Rothamsted, offers one of the few examples of barley sown both bare and undersown with clover. The growth of the undersown barley is perceptibly better during its early stages, and on the two pairs of manured plots (with and without artificial nitrogen) the average yield of barley is higher where it has been undersown. The influence of the general level of nutrition is clearly shown on this field by the poor growth of both barley and clover where no manure is supplied.

Rothamsted experiments provide quantitative information every four years from Agdell Field regarding the effect of clover upon the yield of the cover crop. None of the modern four- and six-course rotation experiments at Rothamsted and Woburn has yet completed a cycle. A one-year's experiment was carried out at Rothamsted in 1931 to compare the effects of no undersown crop, pure clover, pure rye grass, and a mixture of clover and rye grass, all with and without added nitrogen as sulphate of ammonia. The yields of barley tended to be increased by leguminous undersowings (Rothamsted, 1931; Russell and Bishop, 1933).

An extensive search has revealed only one other record of the effect of undersowing legumes in a cover crop. Experiments were performed annually by the Statens Planteavlsudvalg (1917) from 1910 to 1917, with spring oats (in 1910-11 oats mixed with barley) undersown with Italian red clover and with serradella. Nitrogenous manure was applied but no dung. Some results are given in Table I.

Table I. *Yields of cereal grain in kg. per hectare, Statens Planteavlsudvalg, Denmark.*

	Grown alone	Undersown with	
		Serradella	Red clover
Mean yields 1910-16	1004	1052	1062
Yield in wet year 1916	1768	2272	1936

Most agricultural teachers and researchers upon the effect of undersown legumes upon crops (*e.g.* clover, Shutt, 1898; sweet clover (*Melilotus* spp.), Crosby and Kephart, 1931) have devoted their attention to residual manurial effects, ignoring the effect of undersown legumes upon the "nurse" crop. A preponderating manurial action has been ascribed to nitrogen in the dung of the classical four-course rotation. It has not been widely realised that effects of the legume break are traceable during three years out of the four, and that nitrogen (as dung) is carted on to the field only in that year in which sufficient nitrogen manuring from the growth of legumes is not available.

III. TABLES SUMMARISING EXPERIMENTS ON ASSOCIATED GROWTH.

Table II summarises most of the experiments (other than Lipman's and those upon grassland) known to the author which record the effect of growing non-legumes together with a host plant in the absence of added nitrogen. Lyon and

Table II. *Experiments, other than Lipman's, on associated growth, without added nitrogen.*

Made: (a) in U.S.A.; (b) in Finland; (c) at Rothamsted, England.

Season of growth	Host plant	Receptor (non-legume)	Numbers of non-legume plants to 1 legume in mixtures	A* Field P Sand pot S Flask	Authors	Year of publication
1910	Peas	Oats	0.8	A	Lyon and Bizzell (a)	1911
1910	Red clover	Timothy	—	A	Do.	1911
1910	Lucerne	Timothy	—	A	Do.	1911
1924	Soya	Wheat	1.5	P†	Stallings (a)	1926
1927	Peas	Oats	—	P	Virtanen (b)	1928, 1929
1928 and 1929	Red clover	Meadow foxtail	1 and 2	P	Virtanen and v. Hausen (b)	1930, 1931
1929	Peas	Oats	1.5	P	Do.	1930, 1931
1930	Peas	Oats	1	P‡	Do.	1930, 1931
1932	Vetches	Oats	0.33, 1, 3	A	Rothamsted Exp. Sta. (c) (see also Fig. 2 and Table III)	1933
1932	Peas	Oats	0.33	A	Virtanen (b) (Finn. Biochem. Inst.)	1933 a
1932	Peas	Oats	0.41	A	Virtanen (b) (Central Finn. Agric. Org.)	1933 a
	Red clover	Timothy	—	A	Do.	1933 a
1931	Peas	Oats	0.17-4	A§	Vartiovaara (b)	1933
1932	Peas	Oats (2 var.)	—	A	Do.	1933
1932	Peas	Oats	1	S	Virtanen, v. Hausen and Karström (b)	1933
1933	Alder	Birch or pine	—	—	Virtanen and Saastamoinen (b)	1933

* In field experiments (A) the ratios given are not based upon the number of plants but are ratios of weights of seed sown.

† His proportions are not clearly stated in the original.

‡ Experiments at a lower pH than in the preceding.

§ Graphs were constructed giving as abscissae these proportions by weight of seed sown, although the recorded proportions of seedlings varied from 0.9 to 11.2.

Bizzell published no statement regarding manuring of their experiments. Pilz (1911) wrote a pioneer paper of wide scope, which cannot be discussed in this review. Especially brilliant examples of the effects of legumes in mixed vegetation were given by La Flize (1892). (His cordial mention of the work of Petermann apparently refers only to the mode of distribution of manures in soil (Petermann, 1884).)

Other work omitted is that of Bagge (1927), itself a summary of Danish field experiments, and Kellerman and Wright (1914)—a report upon various mixtures grown on nineteen different soils. These authors contended, as did Evans (1916), that considerable benefit resulted, to the non-legume at least, when plants were grown in association. The qualitative observations of Karraker (1925) are of interest.

Ellett, Hill and Harris (1915) thought that the question of benefit from association was open. They are the only investigators to work with plants in greenhouse beds. McClelland (1928), who worked in the dry conditions of Arkansas, suggested that the combination in the field of soya beans or cowpeas with maize was undesirable. It is a common practice to sow such an association in parts of the United States, but McClelland concluded that a true competition for a restricted moisture supply was set up, which was harmful to the growth of maize.

Mooers (1927) stated that in Tennessee soils not moisture but nitrogen was the limiting factor; when cowpeas or soya beans were planted with maize, the yield of maize was depressed. The mixed vegetation gave a larger fodder crop of grain than either crop did if grown singly. The depression of maize yield may nevertheless have been due to competition for moisture on account of the different root habits of the plants. In all of Lipman's (1912) mixtures of maize with cowpeas or soya

Table III. *Experiments on associated growth, with added nitrogen.*

All performed at Rothamsted, England.

Season of growth	Ratios of higher to lowest dressing of mineral nitrogen N/S or S/Amm.†	Legume host plant	Receptor (non-legume)	Numbers of non-legume plants to 1 legume in mixtures	A* Field or P Sand pot	Authors	Year of publication
1930	1, 2, S/Amm.	Peas	Oats	0.58	A	Rothamsted Exp. Sta.	1931
		Vetches	Oats	0.58	A	Do.	
		Peas	Barley	0.80	A	Do.	
		Vetches	Barley	0.80	A	Do.	
1931	1, 3.3, 10, N/S	Lucerne	Italian rye grass	1	P	Thornton and Nicol	1934
1931	1, N/S v. 1, S/Amm.‡	Peas	Oats	0.95	A	Rothamsted Exp. Sta.	1932
		Vetches	Oats	0.95	A	Do.	
		Peas	Wheat	0.95	A	Do.	
		Vetches	Wheat	0.95	A	Do.	
1932	1, S/Amm.	Vetches	Oats	0.33, 1, 3	A	Do. (see Fig. 2)	1933

* In field experiments (A) the ratios given are not based upon the number of plants but are ratios of weights of seed sown.

† N/S = nitrate of soda; S/Amm. = sulphate of ammonia.

‡ With basal manuring with "Adco" compost.

beans, growth of the maize was depressed (cf. Zavitz, 1927). Kaserer (1911) has recorded root interpenetration in mixed crops.

The records of the Cawnpore (1906) experiments, which presumably represent those suggested by Leather (1897), are not informative enough to make discussion profitable.

It will be seen from Table II that Virtanen and his co-workers intend to study the effect of commensalism between two non-legumes, namely, the nodule-bearing *Alnus* and a forest tree. The work of de Peralta and Estioko (1923), on the effects upon rice of the drainage waters from other monocotyledonous plants, should be mentioned.

Table III summarises such experiments upon mixed vegetation (other than grassland) grown with added nitrogenous manures, which provide reliable information upon the transfer of nitrogen from legume to non-legume. None of these was designed to investigate the question of transfer, but they have a bearing upon it. In Denmark, investigations upon the growing of mixed fodder crops have been made since 1899 at least (Anon. 1909). The Rothamsted field experiments all include a comparison of nitrogen with no nitrogen. For other pot experiments with nitrogenous manures added to mixed vegetation see Joulic (1886); Nobbe and Richter (1902); Remy and Vasters (1931), and a graph based upon the results of the last (Thornton and Nicol, 1934). A summary of some work on the subject of associated growth is given by Fred, Baldwin, and McCoy (1932).

IV. CONCLUSION.

It was noted by Raymond Pearl that biologists have been relatively slow to appreciate population factors in the study of living organisms. A single animal has been taken by zoologists to be typical of a species. Bacteriologists give weight to the colonial behaviour of a micro-organism which they are describing, but they study the organism in pure culture, isolated from its fellows, even though it has originated in such a biological complex as soil. Agricultural botanists have been prone to regard competition as something undesirable; probably because they have been mercenarily concerned with the suppression of weeds in otherwise pure cultures of cash crops. In grassland, competition of grasses has been regarded as a nuisance because it is usual for the botanical analysis of a sown sward not to correspond with the percentage composition of the seed mixture sown. These examples no doubt have an anthropocentric basis. Acknowledged authorities have been slow to perceive beneficial effects resulting from "competition." Sir John Lawes was an acute observer, and he was especially interested in the problems of nitrogen nutrition of plants, yet, in his and in Sir Henry Gilbert's published work there is little evidence of any belief that one plant might take up nitrogen from another. Munro and Beaven (1900) had access to Rothamsted records and observations, but in their survey of the effects of clover upon barley in rotation, they imputed such effects to previous, and not to concurrent, leguminous crops. No reference to associated growth has been found in Russell (1932); and benefits

from associated growth were substantially ignored by Clements, Weaver, and Hanson (1929).

Use of the word "competition" is unfortunate, owing to its suggestion of rivalry. It has been shown that activity and efficiency of bacteria in mixed culture with other bacteria (Mahmoud Selim, 1930) and Protozoa (Nasir, 1923; Cutler and Bal, 1926; Meiklejohn, 1930, 1932; Telegdy-Kováts, 1932) are greater than in pure cultures. This is probably true in the soil also. In the case of mixed vegetation just discussed the "competition" between legumes and grasses is essentially a symbiosis. It is, moreover, a double symbiosis in that cereals and grasses profit directly by atmospheric nitrogen fixed by a symbiosis between the leguminous plant and its nodule bacteria. The conditions of a mixed vegetation resemble the case of an ecto-parasite living harmoniously upon a host which itself is nourished through the agency of mycorrhiza. To many, the conception of grassland and of mixed forage crops suggested by this parallel will be new. A true competitive rivalry supervenes only through the agency of man—giving an excess of artificial nitrogen, or by overstocking with grazing animals. The growth of leguminous plants at the expense of non-legumes is to some extent favoured by manuring with carbohydrate, or carbohydrate-rich material (Murray, 1921; Thornton, 1929*b*), but this is probably an effect due to removal of combined nitrogen. Based upon a concept of a population existing not in rivalry, but in harmony, the soundest method of manuring mixed vegetation is to supply abundant phosphate and potash, with lime if necessary, and by thus sustaining and increasing the vigour of the leguminous component, to encourage that double association upon which the natural well-being of the floral population depends.

V. SUMMARY.

About 1840, the beginning of the era of scientific agricultural chemistry, many chemists believed that ammonia was the principal, if not the sole form in which nitrogen was taken up by all plants. This view was abandoned, and towards the end of last century it was generally believed that with the possible exception of the Leguminosae, the higher plants took up their nitrogen almost solely from nitrate. This belief was in large measure founded upon the results of excessive attention paid to the conditions of soil which was not bearing vegetation.

The discovery that leguminous plants were able by the help of specific bacteria to utilise atmospheric nitrogen was not thought to extend to any of the other higher plants. Though widespread use had been made by practical farmers of leguminous plants in association with non-legumes, the idea of commensalism between legumes and non-legumes did not arise amongst agricultural scientists until the present century was well advanced. The acknowledged benefits attained through the growth of legumes were over long ascribed to nitrification of decayed roots resulting from some previous (not to a simultaneous) legume occupancy. This theory may have been correct for the conditions of single crops on arable soils, but was inadequate to account for the comparative failure of grassland and mixed forage crops to respond profitably to fertilising with quickly acting mineral nitrogenous manure.

The rôle of nitrate in the soil is not clearly understood; nitrate is most likely an end-product of micro-organic decomposition of organic materials. Its presence is detectable in notable amounts, and most clearly, in the absence of plants. This does not necessarily imply, as it was once thought, that it is preferentially absorbed by plants; it is suggested that plants can absorb some of the less highly oxidised forms of nitrogen which are the precursors of nitrate. In other words, the finding of nitrates in considerable amounts in soil indicates that there has been a local surplus of nitrifiable nitrogen compounds which plant roots have been unable to reach and consequently to absorb. No single compound of nitrogen can be named as the primary component of the nitrogenous nutrition of plants.

Evidence is presented that non-leguminous plants can profitably utilise compounds of nitrogen built up by the symbiotic life of nodule bacteria within their proper leguminous host plants. Some insight into the nature of the transferred compounds has been gained, though the conditions *in vitro* do not admit of facile extension to natural conditions. The mode of transfer from legume to non-legume is still obscure, but the existence of a transfer can be taken to be well established; it represents a stage in a double symbiosis of which the importance has not been fully appreciated. It is probable that in the nitrogenous nutrition of plants some factors are involved which are not yet formulated. These accessory factors may be found to derive ultimately from the animal, aided by activity of legume nodule bacteria in the soil.

Assistance given by the Imperial Bureau of Soil Science, Harpenden, in permitting a consultation of their card index relating to cover crops, is gladly acknowledged; and thanks are also due to Mr B. Weston, Field Superintendent at Rothamsted, for giving the benefit of his experienced observation to the author.

REFERENCES.

- ANON. (1909). "Resultater af Dyrkningsforsøg med Staldfoderblandinger, saae til forskellig Tid." *Tidsskrift Landbrug. Planteavl*, **16**, 155 (Danish).
 — (1933). "Nitrogen uptake of plants." *Nature*, **131**, 534.
 ARMSTRONG, H. E. (1933). In discussion on lecture by Howard, *q.v.*
 BAGGE, H. (1927). "Forsøg med Vinterblandsæd til Staldfoder 1906–1911 samt Forsøg med forskellige Staldfoderplanter efter Vinterblandsæd 1908–1911." *Tidsskrift for Planteavl*, **33**, 149 (Danish).
 BARRITT, N. W. (1933*a*). "The nitrification process in soils and biological filters." *Ann. Appl. Biol.* **20**, 165.
 — (1933*b*). "Nitrogen and plant nutrition." *Nature*, **132**, 279.
 BEAUMONT, A. B. and MOORE, W. J. (1933). "The absorption and assimilation of ammoniac and nitric nitrogen by plants." *Amer. Fertilizer*, **79**, No. 14, 7.
 BEDFORD, Duke of, and PICKERING, S. U. (1919). *Science and Fruit Growing*. London.
 BLACKMAN, G. E. (1932). "A comparison between the effects of ammonium sulphate and other forms of nitrogen on the botanical composition of closely cut turf." *Ann. Appl. Biol.* **19**, 443.
 BROWN, B. A. (1932*a*). "The effects of fertilization on the chemical composition of vegetation in pastures." *Journ. Amer. Soc. Agron.* **24**, 129.
 — (1932*b*). "Effects of fertilizers on the seasonal production of pastures." *Journ. Amer. Soc. Agron.* **24**, 898.
 Cawnpore Exp. Sta. *Ann. Report* (1906), pp. 30–1.
 CLEMENTS, F. E., WEAVER, J. E. and HANSON, H. C. (1929). *Plant Competition*. Washington, D.C.
 CROSBY, M. A. and KEPHART, L. W. (1931). "Sweetclover in corn belt farming." *U.S. Dept. Agric. Farmers' Bull.* No. 1653.

- CROWTHER, E. M. (1934). Section: "Soils and Fertilisers." *Ann. Rep. Progress Appl. Chem.* (for 1933), p. 519 seq.
- CUTLER, D. W. and BAL, D. V. (1926). "The influence of Protozoa on the process of nitrogen fixation by *Azotobacter chroococcum*." *Ann. Appl. Biol.* **13**, 516.
- CUTLER, D. W. and CRUMP, L. M. (1933). "Some aspects of the physiology of certain nitrite-forming bacteria." *Ann. Appl. Biol.* **20**, 291.
- CUTLER, D. W. and MUKERJI, B. K. (1931). "Nitrite formation by soil bacteria, other than *Nitrosomonas*." *Proc. Roy. Soc. B*, **108**, 384.
- DE PERALTA, F. and ESTIOKO, R. P. (1923). *Philippine Agric.* **11**, 205. Abstr. in *Exp. Sta. Record* (1924), **50**, 832; *Trop. Agriculturist* (1924), **63**, 229.
- DOMONTOVICH, SHESTAKOV and POLOSSIN (1933). "Ueber die Änderung der Wasserstoffionenkonzentration der Nährlösung durch die Tätigkeit der Lupinenwurzeln." *Abh. Soviet. Sekt., Int. Bodenkundl. Gesells. Komm.* **IV**, **2**, 214.
- DORYLAND, C. J. T. (1916). "The influence of energy material upon the relation of soil micro-organisms to soluble plant food." *N. Dakota Agric. Exp. Sta. Bull.* No. 116, 319.
- ELLETT, W. B., HILL, H. H. and HARRIS, W. G. (1915). "The effect of association of legumes and non-legumes." *Va. Agric. Exp. Sta. Bull.* No. 1, 28.
- EVANS, M. V. (1916). "Some effects of legumes on associated non-legumes." *Journ. Amer. Soc. Agron.* **8**, 348.
- FRED, E. B., BALDWIN, I. L. and MCCOY, E. (1932). *Root Nodule Bacteria and Leguminous Plants*. Univ. Wisconsin Studies in Science, No. 5. Madison, Wisc.
- GLAUBER, J. R. (1956). *Des Deutschlands Wohlfahrt*. Amsterdam.
- HALL, A. D., MILLER, N. H. J. and GIMINGHAM, C. T. (1908). "Nitrification in acid soils." *Proc. Roy. Soc. Lond. B*, **80**, 196.
- HARTELIUS, V. (1933). "The occurrence of growth substance B in urine." *Comptes rend. Travaux Lab. Carlsberg*, **19**, No. 18.
- HARTLEY, K. T. and GREENWOOD, M. (1933). "The effect of small applications of farmyard manure on the yield of cereals in Nigeria." *Empire Journ. Exp. Agric.* **1**, 113.
- HESSSELMAN, H. (1925-6). "Studier över barrskogens humustäcke, dess egenskaper och beroende av skogsvården." *Medd. Statens Skogsförsök*, **22**, 169 (Swedish, with German summary).
- HOWARD, A. (1933). "The waste products of agriculture: their utilisation as humus." *Journ. Roy. Soc. Arts*, **82**, 84.
- HUTCHINSON, H. B. and MILLER, N. H. J. (1911). "The direct assimilation of inorganic and organic forms of nitrogen by higher plants." *Centrabl. f. Bakt.* **11**, **30**, 513.
- JASHNOVA, N. V. (1930). "Nitrification in podzol soil." *Trans. Sci. Inst. Fertilisers*, **76**, No. 415, 50.
- JENSEN, H. L. (1932). "The microbiology of farmyard manure decomposition in the soil. III. The decomposition of the cells of micro-organisms." *Journ. Agric. Sci.* **22**, 1.
- JENTSCH, A. (1927). "Die Egartwirtschaft." *Fortschr. der Landw.* **2**, 477.
- JONES, MARTIN G. (1934). "Grassland management and its effect on the nature of the sward." *Scottish Journ. Agric.* **17**, 46.
- JOULIE, H. (1886). "Fixation de l'azote dans le sol cultivé." *Annales agronomiques*, **12**, 5.
- KALATSHIKOV, A. T. (1928-30). "Absorption biologique de l'azote des engrais minéraux: sulfate d'ammonium et nitrate de chaux, dans les sols." *Comptes rend. Lab. Centr. Chim. Agric. Kiev*, p. 132; Abstr. *Ann. agronomiques* (1931), Année **1**, 400.
- KASERER, H. (1911). "Beobachtungen über die Bewurzelung der Kulturpflanzen bei Reinsaat und bei Mischsaat." *Zeitschr. f. d. landw. Vers. Oesterreich*, **14**, 1022.
- KARRAKER, P. E. (1925). "Note on the increased growth of bluegrass from associated growth of sweet clover." *Journ. Amer. Soc. Agron.* **17**, 813.
- KELLERMAN, K. F. and WRIGHT, R. C. (1914). "Mutual influence of certain crops in relation to nitrogen." *Journ. Amer. Soc. Agron.* **6**, 204.
- KUHLMANN, F. (1847). *Expériences chimiques et agronomiques*. Paris.
- KÜLBEL, J. A. (1741). *Cause de la fertilité des terres*. Bordeaux. Quoted by Russell (1932) q.v.
- LA FLIZE, S. (1892). "Expériences sur les Légumineuses." *Ann. Sci. Agron.* **9**, 174.
- LAWES, J. B. and GILBERT, J. H. (1858). "Report of experiments with different manures on permanent meadow land. Part I." *Journ. Roy. Agric. Soc. England*, **19**, 552.
- LEATHER, J. W. (1897). *Final Report of the Agricultural Chemist to the Government of India*, paras. 157-8, p. 99.
- LIPMAN, J. G. (1910a). *Ann. Rep.* for 1909. U.S. Dept. Agric. Office of Exp. Sta. Washington, D.C., p. 150.
- (1910b). "A method for the study of soil fertility problems." *Journ. Agric. Sci.* **3**, 297.
- (1912). "The associative growth of legumes and non-legumes." *New Jersey Agric. Exp. Sta. Bull.* No. 253.
- (1913). "A further discussion of certain methods used in the study of 'The associative growth of legumes and non-legumes'." *Journ. Amer. Soc. Agron.* **5**, 72.

- LYON, T. L. and BIZZELL, J. A. (1911). "A heretofore unnoted benefit from the growth of legumes." *N.Y. Cornell Univ. Agric. Exp. Sta. Bull.* No. 294.
- (1913a). "Is there a mutual stimulation of plants through root influence?" *Journ. Amer. Soc. Agron.* **5**, 38.
- (1913b). "A discussion of certain methods used in the study of 'The associative growth of legumes and non-legumes.'" *Journ. Amer. Soc. Agron.* **5**, 65.
- (1913c). "A reply to J. G. Lipman." *Journ. Amer. Soc. Agron.* **5**, 80.
- MCCARRISON, R. (1926). "The effect of manurial conditions on the nutritive and vitamin values of millet and wheat." *Ind. Journ. Med. Res.* **14**, 351.
- MCCLELLAND, C. K. (1928). "The effect of interplanted legumes on the yields of corn." *Arkan. Agric. Exp. Sta. Bull.* No. 229.
- MAHMOUD SELIM (1930). "A study of nitrogen-fixing bacteria from soils." *Proc. Second Int. Congr. Soil Sci.* **3**, 83. Moscow, 1932.
- MAYOW, J. (1674). *Tractatus quinque medico-physici*. Alembic Club reprint. Edinburgh, 1907.
- MEIKLEJOHN, J. (1930). "The relation between the numbers of a soil bacterium and the ammonia produced by it in peptone solutions; with some reference to the effect on this process of the presence of amoebae." *Ann. Appl. Biol.* **17**, 614.
- (1932). "The effect of *Colpidium* on ammonia production by soil bacteria." *Ann. Appl. Biol.* **19**, 584.
- MOCKERIDGE, F. A. (1920). "The occurrence and nature of the plant growth-promoting substances in various organic manurial composts." *Biochem. Journ.* **14**, 432.
- MOOERS, C. A. (1927). "Influence of cowpea crop on yield of corn. Part II. Effects of cowpeas and soybeans planted with corn." *Tenn. Agric. Exp. Sta. Bull.* No. 137.
- MUNRO, J. M. H. and BEAVEN, E. S. (1900). "Various conditions affecting the malting quality of barley." *Journ. Roy. Agric. Soc. England*, **61**, 185.
- MURRAY, T. J. (1921). "The effect of straw on the biological soil processes." *Soil Sci.* **12**, 233.
- NAKAMURA, T. (1894-7). "On the relative value of asparagine as a nutrient for Phanogams" (*sic*). *Bull. Coll. Agric. Tokyo*, **2**, 465; for Fungi, *id.* 468.
- NASIR, S. M. (1923). "Some preliminary investigations on the relationship of Protozoa to soil fertility with special reference to nitrogen fixation." *Ann. Appl. Biol.* **10**, 122.
- NICOL, HUGH (1932). "Nitre." *Chem. and Ind.* **10**, 971.
- (1933). "Rothamsted experiments on residual values of leguminous crops." *Empire Journ. Exp. Agric.* **1**, 22.
- NOBBE, F. and RICHTER, L. (1902). "Ueber den Einfluss des Nitratstickstoffs und der Humus-substanzen auf den Impfungserfolg bei Leguminosen." *Landw. Vers. Stat.* **56**, 441.
- ONSLow, M. W. (1931). *The Principles of Plant Biochemistry*. Part I. Cambridge.
- PETERMANN, A. (1884). "Recherches sur le meilleur mode d'emploi des engrais artificiels." *Annales agronomiques*, **10**, 241.
- PILZ, F. (1911). "Leguminosen und Gramineen in Rein- und in Mengsaaten mit besonderer Berücksichtigung der Stickstoffausnutzung." *Zeitschr. f. d. landw. Vers. Oesterreich*, **14**, 1150.
- POPOFF, M. (1933). "Ueber die pflanzlichen Auxine und ihre Wirkung auf Einzellige." *Biologisches Zentrbl.* **53**, 661.
- PRIANISHNIKOV, D. N. (1933). "Ueber die äusseren und inneren Bedingungen der Ausnutzung des Ammoniakstickstoffs durch die Pflanzen." *Zeitschr. f. Pflanz. Düng. A.* **30**, 38.
- (1934). "Ueber das Aufschliessen der Rohphosphate durch die Wurzelabscheidungen von Lupinen." *Die Phosphorsäure*, **4**, 1.
- RAMIAH, D. V. (1933). "Organic manures and animal nutrition." *Agriculture and Livestock in India*, **3**, 234.
- REMY, TH. and VASTERS, J. (1931). "Untersuchungen über die Wirkung steigender Stickstoffgaben auf Rein- und Mischbestände von Wiesen- und Weidepflanzen." *Landw. Jahrb.* **73**, 521.
- REUSZER, H. W. (1931). "Microbiological changes occurring in a soil under pasture and bare conditions." *Journ. Amer. Soc. Agron.* **23**, 417.
- RICHARDSON, H. L. (1933). "The behaviour of nitrogenous fertilizers in grassland soils." *Agric. Progress*, **10**, 160.
- ROBINSON, M. E. (1929). "The protein metabolism of the green plant." *New Phytol.* **28**, 117.
- ROTHAMSTED EXP. STATION (AND WOBURN) (1931, 1932, 1933). *Annual Reports for 1930, 1931 and 1932*. Harpenden.
- RUSSELL, E. J. (1932). *Soil Conditions and Plant Growth*. London.
- RUSSELL, E. J. and BISHOP, L. R. (1933). "Investigations on Barley. Report on ten years of experiments." *Suppt. Journ. Inst. Brewing*, **39**, 287.
- SHUTT, F. T. (1898). *Canada Exp. Farms Report for 1897*, pp. 138-40. Ottawa.
- SKINNER, J. J. and NOLL, C. F. (1919). "Botanical composition of a permanent pasture as influenced by fertilizers of different compositions." *Soil Sci.* **7**, 161.
- STALLINGS, J. H. (1926). "The form of legume nitrogen assimilated by non-legumes when grown in association." *Soil Sci.* **21**, 253.

- [STATENS PLANTEAVLSUDVALG.] (1917). "Forsøg med Dyrkning i Havre af Serradella og Tidlig Rødklover til Grøngødning, 1910-16." *Tidsskrift Landbrug. Planteavl*, **24**, 614 (Danish).
- TELEGDY-KOVÁTS, L. DE (1932). "The growth and respiration of bacteria in sand cultures in the presence and absence of Protozoa." *Ann. Appl. Biol.* **19**, 65.
- THORNTON, H. G. (1929*a*). "The 'inoculation' of Lucerne (*Medicago sativa* L.) in Great Britain." *Journ. Agric. Sci.* **19**, 48.
- (1929*b*). "The effect of fresh straw on the growth of certain legumes." *Journ. Agric. Sci.* **19**, 563.
- THORNTON, H. G. and NICOL, HUGH (1934). "The effect of sodium nitrate on the growth and nitrogen content of a lucerne and grass mixture." *Journ. Agric. Sci.* **24**, 269.
- THORNTON, H. G. and SMITH, G. (1914). "On the nutritive conditions determining the growth of certain fresh-water and soil protista." *Proc. Roy. Soc. Lond. B*, **88**, 151.
- TYAGNY-RYADNO, M. (1933). "The relations of *Bacillus mycoides* with ammonification, nitrification, and soil fertility." *Journ. Agric. Sci.* **23**, 335.
- VARTIOVAARA, U. (1933). "Untersuchungen über die Leguminosen-Bakterien und -Pflanzen. XIII. Ueber den Stickstoffhaushalt des Hafers bei feldmässigen Mischkulturen zusammen mit der Erbse." *Zeitschr. f. Pflanz. Düng. A*, **31**, 353.
- VIRTANEN, A. I. (1928). *Biochem. Zeitschr.* **193**, 300.
- (1929). "Undersøgelser over Bælplantebakterierne og Bælplanterne." *Beret. Nordiske Jordbrugs Forenings, IV Kongres* (Helsingfors), p. 782 (Danish). København.
- (1933*a*). "Investigations on the root nodule bacteria of leguminous plants. XIV. The inoculation of leguminous crops in the light of field experiments." *Kemiantutkimus-Säätiön biokemiallisen Laboratorion julkaisu*, No. 1 (Finnish, with English summary).
- (1933*b*). "Ueber die Stickstoffernährung der Pflanzen." *Ann. Acad. Sci. Fenn. A*, **36**, No. 12 (German).
- VIRTANEN, A. I. and VON HAUSEN, S. (1930). "The capability of grass plants to take advantage of nitrogen fixed by the nodule bacteria of leguminous plants." *Contrib. Lab. Valio, Helsinki* (Finnish, with English summary).
- (1931). "Untersuchungen über die Leguminosen-Bakterien und -Pflanzen. X. Ueber die Tätigkeit der Leguminosenbakterien und die Ausnützung des in Wurzelknöllchen der Leguminosen gebundenen Stickstoffs durch Nicht-Leguminosen." *Zeitschr. f. Pflanz. Düng. A*, **21**, 57.
- VIRTANEN, A. I., VON HAUSEN, S. and KARSTRÖM, H. (1933). "Untersuchungen über die Leguminose-Bakterien und -Pflanzen. XII. Die Ausnützung der aus den Wurzelknöllchen der Leguminosen herausdiffundierten Stickstoffverbindungen durch Nichtleguminosen." *Biochem. Zeitschr.* **258**, 106.
- VIRTANEN, A. I. and SAASTAMOINEN, S. (1933). "Ueber die Stickstoffbindung bei Erlen (*Alnus*)." *Acta Chem. Fenn. B*, **6** (German).
- VISVA NATH, B. (1931). "Food requirements of crops with reference to South Indian soils." *Ind. Journ. Agric. Sci.* **1**, 495.
- (1932). *Some aspects of plant nutrition*. Soc. Biol. Chemists, India. Bangalore.
- VISVA NATH, B. and SURYANARAYANA, M. (1927). "The effect of manuring a crop on the vegetation and reproductive capacity of the seed." *Mem. Dept. Agric. India, Chem. Ser.* **9**, 85.
- WALLERIUS, J. G. (1761). *Agriculturae Fundamenta Chemica*. Upsala.
- WILLIS, L. G. and RANKIN, W. H. (1930). "Free-ammonia injury with concentrated fertilizers." *Journ. Ind. Eng. Chem.* **22**, 1405.
- WOOD, J. G. (1933). "The nitrogen metabolism of the leaves of *Atriplex nummularium*." *Austral. Journ. Exp. Biol.* **11**, 237.
- ZAVITZ, C. A. (1927). "Forty years' experiments with grain crops." *Ont. Agric. Coll. Dept. Agric. Bull.* No. 332.

ADDENDA.

A paper by H. C. Trumble on "The agricultural features of *Phalaris tuberosa* and allied forms" (*Journ. of Agric. South Australia* (1933), **37**, 400) contains the novel postulate that for sustained productivity of *P. tuberosa* "a suitable associate legume" is desirable. A further paper by H. C. Trumble and J. G. Davies is announced, in which will be given "evidence of the value of legumes in association with permanent grasses." J. H. Gurski has compared oats-barley and oats-vetch mixtures. *Doświadc. Roln.* (1927), **3**, Parts (Części) III-IV, 55. Warszawa.

FURTHER EVIDENCE UPON THE NITROGEN UPTAKE OF GRASS GROWN WITH LUCERNE.

BY H. G. THORNTON AND HUGH NICOL.

(*Rothamsted Experimental Station, Harpenden, Herts.*)

(With One Text-figure.)

THE practice of growing mixed crops of legumes and non-legumes is widespread. There is reason to believe that clovers growing with grasses in a pasture do not merely act as a direct source of protein for animals, but also contribute to the nitrogenous intake of the grasses. The earliest reference to possible benefit derived by a non-legume from a legume growing beside it appears to have been made by Leather (1897), though Sinclair (as quoted by Wilson (1848) in a reference that has not been traced by the authors) had noted that a mixture of many grasses grew better than only one or two species. Sinclair (1816), like eighteenth-century writers, used the term *grass* in a wide sense to include leguminous pasture plants. The first experimental demonstration of a non-leguminous plant grown with only that nitrogen supplied by a leguminous plant was made by J. G. Lipman (1910). A review of the available literature upon the excretion of nitrogenous compounds by legumes has been made by one of us (Nicol, 1934), and it will therefore suffice here to state that the reality of a gain of nitrogen by a non-legume when grown in association with a legume seems to have been established, though the mechanism is still obscure.

In 1931 the authors carried out an experiment wherein lucerne and Italian rye-grass were grown together in pots in sand. Sodium nitrate was used at three low levels, namely 0.33, 1.0 and 3 gm. per 20 kg. of sand, one dose being added to each pot at the time of sowing the seed. The results of the growth of grass, as shown in Fig. 1, indicated that considerably more nitrogen than had been supplied was found in the grass from those pots which had received the two smallest doses (*AA* and *BB*) of nitrate. The recovery varied from two to nearly six times the initial dose of nitrogen, even on the assumption that none of the nitrate had been taken up by the lucerne.

The results for the grass in 1931 have already been given in detail (Thornton and Nicol, 1934 *a*) both as regards dry weight and nitrogen content, but the figures for nitrogen content are repeated in graphical form in Fig. 1 for the sake of comparison with the 1933 results.

In 1933 similar mixtures of lucerne and rye-grass were grown, but to these no nitrogenous manure was supplied. Whereas in the 1931 experiment no grass was grown by itself, in 1933 four pots were sown with grass only. In respect of greenhouse technique the experiments were similar (for details see Thornton and Nicol (1934 *a*, 1934 *b*). In both years harvests were made at approximately regular intervals throughout the growing season, except that the grass grown by itself was not harvested until the time of making the last harvest of the mixture of lucerne and grass. Harvesting implied the turning out of the pots and, usually, separation of roots and tops of each species, though in 1933 the grass roots and tops were analysed together.

The 1933 experiment also provided information upon the effects of clipping or cutting the lucerne tops. The effects of clipping upon the lucerne component of the mixture have been discussed elsewhere (Thornton and Nicol, 1934 *b*). This paper is concerned only with the effects of lucerne, clipped (in 1933) and unclipped (in 1931 and 1933), upon the growth of the grass.

Results of the harvesting of grass grown alone and with clipped and unclipped lucerne are presented in Table I. Each determination in the table is an average obtained from the number of parallel pots shown.

Table I. *Growth of grass (tops and roots), sown with and without lucerne, on April 20th, 1933 (Rothamsted).*

Date of harvest, 1933	June 14th	July 6th	July 27th		August 24th		August 24th
With lucerne ...	Un- clipped	Un- clipped	Un- clipped	Clipped	Un- clipped	Clipped	Grown alone
<i>Number of parallels</i>	8	6	6	5	6	5	4
Dry matter, gm.	0.38	0.86	1.2	1.4	4.5	6.9	5.15
Nitrogen, %*	0.84	0.93	0.86	0.92	0.86	0.65	0.33
Total nitrogen, mg.	3.1	7.7	10.1	12.7	37.1	40.2	16.5
	±0.44	±0.60	±0.74	±1.28	±5.94	±3.50	±0.26

* Unweighted means.

The grass at the first harvest contained only 3.1 mg. of nitrogen. By August 24th the grass grown alone contained 16.5 mg., the difference being presumably derived either from impurities in the sand or from nitrogen fixed by free-living bacteria. The presence of lucerne further increased the nitrogen content of the grass to 37 and 40 mg. by August

24th, that is in about 4 months. It appears probable from its rate of nitrogen increase, that the grass grown with lucerne did not contain appreciably more nitrogen than that grown alone until after July 27th, or, in other words, that the uptake by grass of nitrogen from lucerne in the absence of added nitrogen began after about 3 months from sowing.

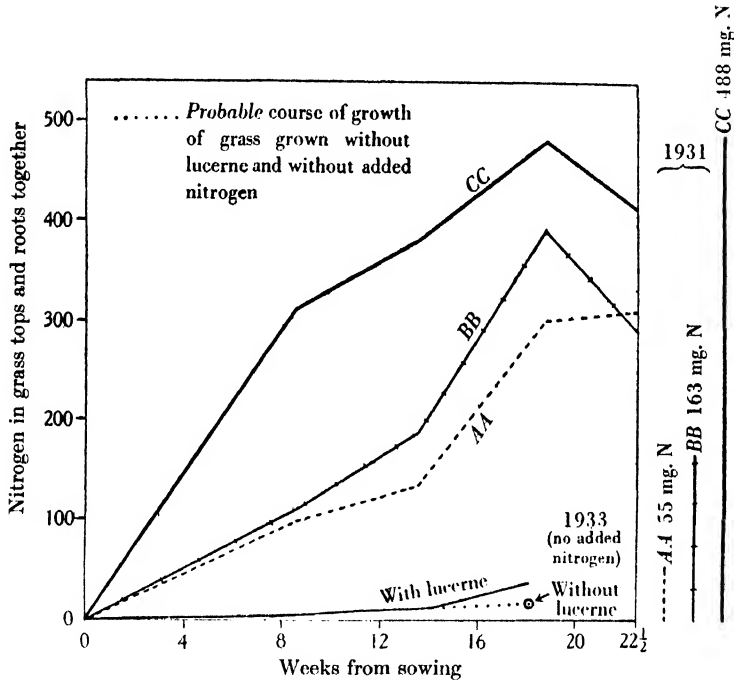


Fig. 1. Milligrams of nitrogen in grass (roots and tops together). The three uppermost curves indicate the course of nitrogen uptake in 1931 of grass grown in presence of lucerne; three levels of nitrogen as sodium nitrate were added in one dose at the time of sowing. The doses of nitrogen are represented to the same vertical scale and with the same kind of lines at the right of the figure. The dot represents the nitrogen content of grass grown in 1933 in absence of both lucerne and added nitrogen, while the thin line near the bottom shows the nitrogen content of grass grown with lucerne in absence of added nitrogen. For this curve were taken the means of the results from grass grown with clipped and unclipped lucerne. Each point represents the mean of results of from three to eight parallel pots in which the plants were grown in sand.

There was probably no significant effect upon grass of clipping the lucerne. In this experiment, therefore, where no nitrogenous manure was given, the uptake of nitrogen by the grass from the lucerne appears to have begun later than was the case in the 1931 experiment where small doses of nitrate were applied. It seems likely that such small doses of nitrate, by encouraging the early root development of the grass,

increased its capacity for absorbing nitrogen compounds from the lucerne.

The authors have made no attempt to trace out the course of the supposed transfer of nitrogen from legume to non-legume. Virtanen (1933 *a, b*) has recorded finding in pure sand, in which inoculated legumes were grown, amounts of combined nitrogen of the same order as that existing in the plants themselves. Wilson, of Madison, Wisconsin (1934), stated that he has tried to repeat the technique of Virtanen and his co-workers, so far without being able to confirm their findings regarding the excretion of nitrogenous compounds by leguminous plants.

SUMMARY AND ABSTRACT.

1. Italian rye-grass grown in the presence of lucerne in sand with no added nitrogen contained, after 18 weeks' growth, some $2\frac{1}{4}$ times as much nitrogen as did grass of the same age similarly grown but in the absence of lucerne.

2. In another experiment, where lucerne and grass were grown together in sand:

(a) Where 0.33 gm. of sodium nitrate was added per pot, the grass after only $13\frac{1}{2}$ weeks' growth contained $2\frac{1}{2}$ times, and after 18 weeks' $5\frac{1}{2}$ times, as much nitrogen as was supplied as nitrate.

(b) Where 1.0 gm. of nitrate was added, the grass after $13\frac{1}{2}$ weeks contained slightly more nitrogen than was added as nitrate, and after 18 weeks it contained $2\frac{1}{4}$ times as much.

(c) Where 3.0 gm. of nitrate were supplied, there was slightly less than a quantitative recovery in the grass of the nitrate nitrogen supplied.

REFERENCES.

- LEATHER, J. W. *Final Rep. Agric. Chemist to Govt. of India* (1897), paras. 157-8, p. 99.
 LIPMAN, J. G. *Ann. Rep. for 1909* (1910), U.S. Dept. Agric., Office of Exp. Sta., Washington, D.C., p. 150.
 NICOL, HUGH. *Biol. Rev.* (1934), **9**, 383.
 SINCLAIR, G. *Hortus Gramineus Woburnensis* (1816). London. Also later editions.
 THORNTON, H. G. and NICOL, HUGH. *J. Agric. Sci.* (1934 *a*), **24**, 269.
 ——— *J. Agric. Sci.* (1934 *b*), **24**, 532.
 VIRTANEN, A. I. *Ann. Acad. Sci. Fenn. Ser. A* (1933 *a*), **36**, No. 12. Anonymous summary in *Nature* (1933), **131**, 534.
 ——— *Herbage Reviews* (1933 *b*). (Aberystwyth), **1**, 88.
 WILSON, J. M. *The Rural Cyclopaedia*, Edinburgh (1848), **2**, 498, article "Grasses."
 WILSON, P. W. Private communication (1934).

(Received June 5th, 1934.)

SOME EFFECTS OF CLIPPING THE TOPS UPON THE ROOT DEVELOPMENT OF LUCERNE (*MEDICAGO SATIVA* L.).

BY H. G. THORNTON AND HUGH NICOL.

(*Bacteriology Department, Rothamsted Experimental Station,
Harpenden, Herts.*)

(With One Text-figure.)

I. INTRODUCTION.

THE problem of cutting lucerne in the seedling year is one that has been much discussed. As long ago as the eighteenth century B[artholomew] Rocque⁽¹⁾, of London, wrote as follows:

“In Languedoc they sow no Corn with it: And, when the Lucern is six or seven Inches high, they mow it; by which all the Seeds of annual Weeds are cut off and killed.

“By my own Experience, I find they are in the wrong; for it bleeds it, so that the Plants make but little Progress. . .”

The second paragraph occurs in the second edition (1765) only, suggesting that the author had had several years' experience of the harmful effect of cutting lucerne too early. Rocque's opinion, however, was far from meeting with universal acceptance. Indeed, the view that early cutting encouraged the vitality of young lucerne persisted in America until the beginning of the present century; for some typical opinions see McKee⁽²⁾ and Graber *et al.*⁽³⁾ At the present day, however, it is widely recognised among American agronomists that excessive cutting in the seedling year is harmful.

Sundelin and Franck⁽⁴⁾ concluded from experiments in Sweden also, that lucerne should not be cut during the year of sowing. They sowed lucerne in mid-June in successive years and examined the effects of not cutting in the year of sowing and of cutting after about 61, 83 and 100 days, recording the yield (of two cuts) in following years. Marked depressions in yield followed after the late cuts, the extent of the effects varying with season. Early cuttings in the year of sowing were performed in August, and so enabled the plants to recover before winter. From the results of Sundelin and Franck it appears that the effects of

cutting very young plants were largely offset when the plants were allowed to recuperate before the onset of winter. In 1922 late autumn cutting of newly-sown lucerne prejudiced the yield for three years afterwards. In the case of lucerne sown in 1923, the cut, whenever taken in that year, severely depressed the yields of 1924 and 1925. Sundelin and Franck refer, however, to June sown lucerne, upon which the effect of cutting the young plant may be more harmful than upon lucerne sown in spring as seems desirable in this country.

The results of cutting are somewhat complex and it is important to distinguish its influence upon the plant's food reserves from secondary effects, as upon weed infestation on the one hand and on the other hand upon possible reduction in autumn growth and in the consequent winter covering. This distinction is of practical importance, since the magnitude of these various effects is much influenced by the time as well as the frequency of cutting. The present work deals with the influence of clipping upon the plant's food reserves.

In the case of a nodule-bearing legume, cutting might affect the food reserves in two ways, either by its direct influence upon the materials translocated to or from the roots, or, indirectly by influencing nitrogen fixation in the nodules. These two effects can be distinguished by comparing the mass and composition of roots and tops in clipped and unclipped plants on the one hand and on the other by estimating the effect of clipping¹ upon the total nitrogen fixed. The literature records very few studies of the effect of cutting lucerne upon its root development, although, as pointed out by Graber(5), its clear-cut responses to cutting treatments make this plant valuable as a medium for initial investigations on root reserves. A review has been made by Nicol(12).

Graber wrote a largely theoretical paper on the growth of lucerne with various cutting treatments. His tentative conclusions appear sound in the light of later work(3, 6, 7, 8). Late or excessive cutting, according to Graber, leads to (1) smaller root reserves, (2) lessened absorptive capacity of the roots, (3) increased winter killing, (4) increased invasion by weeds owing to encroachment by them upon spots left bare by winter killing. Definite figures relative to the effect of clipping upon thickness of stand, as well as upon the diameter of roots, of lucerne plants were given by McKee(2); some of his results are summarised in Table I. Other measurements of root diameters of clipped and unclipped lucerne, performed by later workers (9, 10), have borne out the conclusions

¹ The term "clipping" is here used to designate the equivalent in pot culture work to cutting or mowing in the field.

Table I. *Effect of clipping in year of sowing upon number of lucerne plants and diameter of lucerne roots. Data of McKee(2).*

	Measurements made in	Treatment	Total number of plants	Average diameter of roots mm.
Sown in rows	1912	Clipped	116	7.49
	1912	Not clipped	104	10.48
	1913	Clipped	152	10.98
	1913	Not clipped	218	12.90
Sown broadcast	1913	Clipped	1619	6.56
	1913	Not clipped	1829	6.91
	1914	Clipped	977	6.34
	1914	Not clipped	1013	6.93

suggested by McKee's figures. A review of several papers on cutting, root development, and winter killing has been given by Kiesselbach and Anderson (11). A small reduction in mean root diameter probably reflects a considerable diminution in total mass of root but, since the correlation between these two measurements is imperfectly known¹ and probably variable, the conclusions to be drawn from root diameters are at best somewhat uncertain. The authors have been unable to find any published data from which it is possible to determine whether cutting affects the quantity of nitrogen fixed or merely alters the distribution of nitrogenous and other food materials between the roots and tops. They therefore undertook in 1933 an investigation of the effects of cutting young lucerne once and twice, with a view to obtaining a measure of the root development and nitrogen content of cut and uncut lucerne, grown in sand culture in glazed pots.

II. PLAN OF EXPERIMENT.

The experiment was carried out in an unheated greenhouse. Thirty-six large glazed pots each holding about 20 kg. of clean river sand and watered with nitrogen-free food solution were sown with lucerne and Italian rye-grass on April 20th. Four harvests² were made at intervals of about three weeks, namely on June 14th (set A), July 6th (set B), July 27th (set C) and August 24th (set D). Up to July 5th all pots received the same treatment, but on that date the lucerne in ten of the pots was clipped. The crop in five of these pots was harvested on

¹ Nelson⁽¹⁰⁾ concluded from measurements of over 200 lucerne plants that the green weight of the roots was closely correlated with "the squares of the root diameters near the crown."

² Throughout this paper the terms "harvest" and "harvesting" refer to the turning out of the roots and sand from the pots.

July 27th together with that from six pots in which the lucerne had not been clipped, the eleven pots comprising series C. The remaining five pots of clipped lucerne were again clipped on July 26th and harvested together with six pots of unclipped lucerne on August 24th (series D). Table II shows the dates of clipping and of harvesting (*i.e.* washing the roots) for each set of pots.

Table II.

	Harvest set					
	A	B	C		D	
Date of harvest	June 14	July 6	July 27		August 24	
Dates of clipping	Unclipped	Unclipped	Unclipped	July 5th	Unclipped	July 5th July 26th
Number of replicate pots	8	6	6	5	6	5

III. TECHNIQUE.

On April 20th, 1933, about twenty-five seeds of Grimm lucerne, "inoculated" with an active nodule-forming strain of lucerne nodule bacteria, were sown in each pot. Each seed was placed at the bottom of a small depression made in moist sand and later covered with the surrounding sand. On the day prior to sowing every pot received 500 ml. of food solution (0.5 gm. each of K_2HPO_4 , KH_2PO_4 , NaCl, $MgSO_4 \cdot 7H_2O$, and $CaSO_4$, made up to 500 ml. with rain water with the subsequent addition of 0.04 gm. "anhydrous" ferric chloride). Further quantities, uniform for all pots at one time, of food solution and of rain water were added as thought necessary throughout the period of the experiment. No nitrogenous manure was added at any stage.

In addition to the lucerne seed, about twenty-five seeds of Italian rye-grass were sown in each pot, and a further four pots were sown with the rye-grass alone. The grass, however, made very little growth, and this was scarcely affected by the clipping treatment. Randomisation was adopted throughout, as regards the position in the greenhouse of the various treatments (clippings) and of the pots within each treatment, and as regards the taking of pots for harvesting. Pots were harvested after allowing them to remain unwatered for a few days so that they dried out; then the contents were emptied on to a coarse sieve and the plants (lucerne and grass) separated as completely as possible. Counts of nodules in each pot were made and from 50 to over 200 nodules per pot were measured for length. In all but the first harvest of lucerne, roots and tops were separately dried and weighed and analysed for

nitrogen. The first harvest of lucerne, and the grass throughout, were weighed and analysed for nitrogen as whole plants. Clippings were made with a pair of scissors using eye judgment only, so as to leave about 10 cm. of stem. The clippings from the lucerne were dried, weighed and analysed for nitrogen apart from the rest of the lucerne plants. Those pots in which the lucerne had previously been cut were harvested similarly to the other pots. Those four pots containing grass only were harvested at the end of the experiment (August 24th). All dry weights of lucerne roots and tops, of lucerne clippings, and of grass, were taken in the air-dry state after exposure in a drying room. Analyses were performed by the Chemistry Department of this Station upon the air-dried samples after they had been ground to a fine meal. Dry weights and analyses were obtained separately from each pot, but only the means of the replicates are presented in the tables following. The agreement between replicate pots was good, especially so in the case of the clippings from standing lucerne.

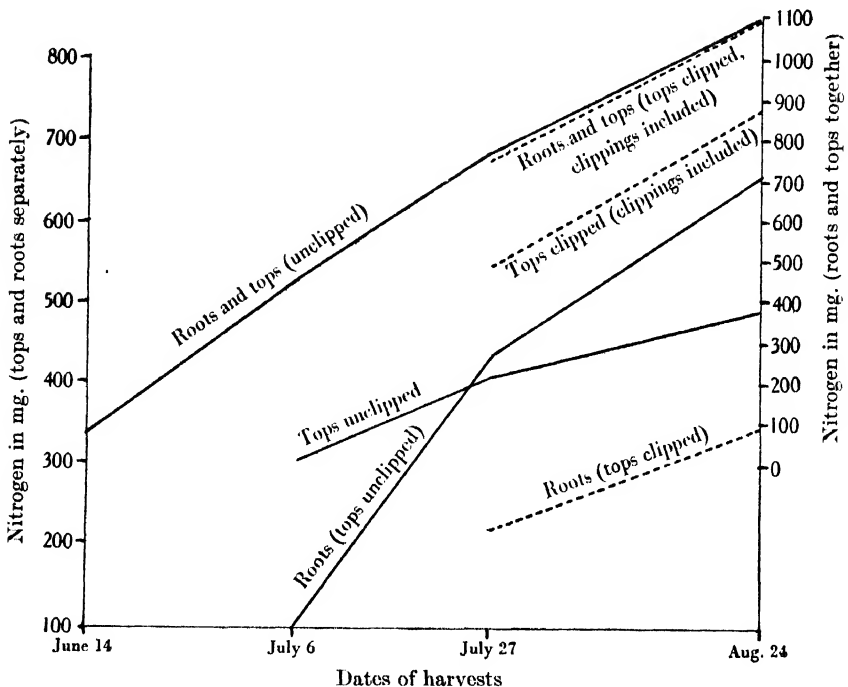


Fig. 1. Influence of clipping the tops of lucerne upon the nitrogen distribution in the plant.

IV. RESULTS.

The results of the experiment are summarised in Tables III and IV and in Fig. 1, where the data quoted represent means of the replicate pots the number of which are given in Table II. Standard errors of these means are italicised in Table III for sets C and D.

Table III. *Nodule numbers, dry weights and total nitrogen in clipped and unclipped lucerne (sown April 20th).*

Set	A	B	C			D		
Date of harvest	June 14th	July 6th	July 27th			August 24th		
Dates of clipping	Un-clipped	Un-clipped	Un-clipped	Clipped July 5	Difference	Un-clipped	Clipped July 5 and July 26	Difference
Number of nodules per pot			294	932	1222	1147	-75	898	1000	+102
Mean nodule length, mm.			2.26	1.65	2.46	2.36	-0.1	2.88	2.73	-0.15
Dry matter of lucerne in gm.:										
Tops			—	10.37	17.74 <i>±0.33</i>	18.49* <i>±0.79</i>	+0.75	20.42 <i>±0.50</i>	24.47† <i>±0.90</i>	+4.05
Roots			—	5.82	16.50 <i>±0.46</i>	10.33 <i>±1.94</i>	-6.17	24.12 <i>±0.91</i>	16.04 <i>±1.51</i>	-8.08
Total			2.29	16.19	34.24 <i>±0.62</i>	28.82* <i>±2.09</i>	-5.42	44.54 <i>±1.05</i>	40.51† <i>±2.39</i>	-4.03
Nitrogen in lucerne in mg.:										
Tops			—	301.1	419.1 <i>±15.81</i>	548.3* <i>±16.45</i>	+129.2	489.8 <i>±23.53</i>	741.9† <i>±31.02</i>	+252.1
Roots			—	152.0	380.3 <i>±10.33</i>	222.2 <i>±30.35</i>	-158.1	606.4 <i>±22.97</i>	343.3 <i>±122.7</i>	-263.1
Total			71.5	453.1	799.4 <i>±21.54</i>	770.5* <i>±43.90</i>	-28.9	1096.2 <i>±28.34</i>	1085.2† <i>±68.51</i>	-11.0

* Including clipping: dry weight 6.87 gm., N 228.1 mg.

† Including first clipping: dry weight 6.98 gm., N 233.2 mg.; second clipping: dry weight 6.84 gm., N 243.2 mg.

Table IV. *Percentages of nitrogen in clipped and unclipped lucerne.*

Set	A	B	C		D	
Date of harvest	June 14th	July 6th	July 27th		August 24th	
Clipping	Un-clipped	Un-clipped	Un-clipped	Clipped	Un-clipped	Clipped
<i>In:</i>								
Roots			—	2.62	2.31	2.15	2.52	2.12
Standing tops, at date of harvest			—	2.91	2.36	2.77	2.48	2.41
First clipping, July 5th			—	—	—	3.36	—	3.35
Second clipping, July 26th			—	—	—	—	—	3.55
Mean for whole plant plus clippings, if any			3.11	2.80	2.34	2.67	2.46	2.68

The unclipped lucerne showed a steady increase in dry weight and nitrogen content both as regards tops and roots during the growing period.

The young tops harvested in June and the clippings taken in July were richer in nitrogen than were the more stemmy attached tops harvested in July and August. The total nitrogen content of the plants (roots and tops including clippings) was not significantly affected by clipping. This did not therefore affect the activity of the nodules. It was also without significant effect upon their numbers and mean size. There was, however, a striking translocation of nitrogen from roots to tops in the clipped plants. In series C, the single clipping increased the nitrogen content of the tops, *plus* clippings, by 129.2 mg., a gain of 30.8 per cent. over the unclipped series, but it reduced the nitrogen in the roots by 158.1 mg., a loss of 41.6 per cent. In series D, the two clippings raised the nitrogen content of the tops by 252.1 mg. (51.5 per cent.), but reduced that of the roots by 263.1 mg. (43.4 per cent.).

Clipping produced a comparatively slight increase in the dry weight of the tops, *plus* clippings, over that of the unclipped tops, but it resulted in so considerable a reduction in the dry matter of the roots that the total dry weight of the whole plant, roots, standing tops and clippings, was appreciably reduced in series C and less so in series D. Since this reduction in total dry matter did not involve the nitrogen it must have concerned the carbon and consequently have represented a lessened photosynthesis or else a more rapid oxidation of carbon by the plant or by the bacteria in the nodules.

V. SUMMARY AND ABSTRACT.

1. Inoculated lucerne was grown in pots of sand and watered with nitrogen-free food solution. In some of the pots the lucerne was clipped once, in some twice and in some it was left unclipped. Pots were harvested on four dates at intervals of about three weeks. Counts and measurements of nodules were made and dry weights and nitrogen contents of tops and roots were obtained.

2. Clipping did not significantly alter the nodule numbers, their mean size, or the total nitrogen contents of the plants, *i.e.* in tops, including clippings, *plus* roots.

3. Clipping, however, resulted in a decrease in the nitrogen content of the roots of about 40 per cent. as compared with unclipped plants. This nitrogen was transferred to the tops where it was removed in the clippings.

4. In clipped plants the total yield of the tops, including clippings, was slightly increased but that of the roots was greatly depressed. This resulted in a reduction in total dry weight of the whole plants.

VI. ACKNOWLEDGMENT.

The authors wish to record their thanks to Miss Sheila Arnold for her assistance in the greenhouse and in making weighings in the laboratory.

REFERENCES.

- (1) ROCQUE, B. *A Practical Treatise of Cultivating Lucern*. London: 1761. *A Practical Treatise on Cultivating Lucern Grass*. London: 1765. (Both citations from 1765 edition.)
- (2) MCKEE, R. *J. Amer. Soc. Agron.* (1916), **8**, 329.
- (3) GRABER, L. F., NELSON, N. T., LUEKEL, W. A. and ALBERT, W. B. *Wisc. Agric. Exp. Sta. Research Bull.* No. 80 (1927).
- (4) SUNDELIN, G. and FRANCK, O. *Centralanstalt. f. försöksv. på jordbruksomr.* Stockholm (1928), Medd. No. 335.
- (5) GRABER, L. F. *J. Amer. Soc. Agron.* (1924), **16**, 169.
- (6) GRANDFIELD, C. O. *J. Amer. Soc. Agron.* (1934), **26**, 179.
- (7) JANSSEN, G. *J. Amer. Soc. Agron.* (1929), **21**, 895.
- (8) ALBERT, W. B. *J. Amer. Soc. Agron.* (1927), **19**, 624.
- (9) SALMON, S. C., SWANSON, C. O. and McCAMPBELL, C. W. *Kansas Agric. Exp. Sta. Techn. Bull.* (1925), No. 15.
- (10) NELSON, N. T. *J. Amer. Soc. Agron.* (1925), **17**, 100.
- (11) KIESSELBACH, T. A. and ANDERSON, ARTHUR. *Univ. Nebr. Agric. Exp. Sta. Research Bull.* (1926), No. 36.
- (12) NICOL, HUGH. Yield, duration, and drought-resistance of lucerne as influenced by frequency and time of cutting. *Emp. J. Exp. Agric.* (In the press.)

(Received May 29th, 1934.)

YIELD, DURATION, AND DROUGHT-RESISTANCE OF LUCERNE AS INFLUENCED BY FREQUENCY AND TIME OF CUTTING

HUGH NICOL

(Department of Bacteriology, Rothamsted Experimental Station, Harpenden)

THE problem of securing the most advantageous yield from a perennial forage crop is peculiarly subtle. It is not easy to suggest how a maximum yield may be obtained; and in addition to mere tonnage, several factors have to be considered, such as convenience of the farmer, seasonal requirements of the stock, digestibility of the fodder, and the influence of cutting upon future yields in the same year and upon the number of years that the crop may be expected to stand.

These requirements are to some extent antagonistic. It now seems probable that the most economic time to cut an established crop of lucerne either for hay or for green fodder so as to secure the best yield of a season is when the plants are in early bloom, when fresh shoots are breaking from the crown. Thornton and Nicol [1] recommended watching for the development of new shoots from the crown as the best indication of readiness for cutting. This recommendation was based upon the conclusions of many experimenters in the United States, but it was admitted that experimental evidence was then lacking for conditions in Great Britain (Woodman [2]). Even in the United States, where the lucerne crop has been most extensively studied, few experiments have been made to examine the effects of a season's cutting upon the yields of subsequent years; the majority of the experiments which have been performed upon the cutting of lucerne have considered digestibility together with bulk, obtainable in one year, usually after the seedling year. Very few records of experiments have been found by the author bearing directly on the effect of cutting in the year of sowing, and only a few others have been found in which the effects in subsequent years of cutting established lucerne were determined. Of these various publications, three are Scandinavian; and they appear to be little known. The language difficulty may therefore serve as the principal excuse for this review.

The problem of summarizing experiments upon the after-effects of cutting lucerne is made difficult by the necessity for distinguishing at least the year of sowing from later years. The diversity of cultural practices employed during the first few months of the crop leads to the subjection of crops of nominally the same age to very different influences, apart from cutting. Lucerne is sown at any time from spring to autumn, and when sown in spring and early summer it may be sown either on bare land or on land already occupied by a corn crop. It is not surprising that testimony to the utility and ease of management of lucerne should vary, since experimental evidence regarding the wisdom of any particular cultural practice or combination of practices is lacking. Thornton [3] was able to clear up one point when he showed that there was frequently

a benefit from inoculating lucerne seed when the crop was intended to be sown under a cereal. Regarding many other points in the cultivation of lucerne, there is no help but traditional experience varying over a wide range with but a relatively limited acreage of crop. Discussion of results is difficult in respect of cutting: two lucerne crops cut three months after sowing are differently fitted to stand the winter according as they had been sown in April or in June. Unfortunately, in the literature, details of the history of experimental crops are often insufficient.

1. The Immediate Effects of Clipping: in the Season of Sowing

Without attempting the operose task of carrying out field experiments to test all the possible combinations of conditions, Thornton and Nicol [4] devised a one-season's greenhouse experiment upon the effects of cutting of lucerne upon the roots of the plants. The results already published show that cutting (or rather its greenhouse equivalent, clipping) seedling lucerne twice, resulted in marked arrest of root-development. The total yield of dry matter and total nitrogen recovered in roots, standing tops, and clippings was not significantly affected by the two cuttings; the apparent net result of clipping was to transfer dry matter from root to top. The clippings were more nitrogenous, and therefore presumably less fibrous and more digestible, than the unclipped tops. A slightly larger yield of dry matter in tops was obtained by clipping than by allowing the lucerne to grow on. These advantages, however, were obtained wholly at the expense of the roots. Haselhoff, quoted by Walther [5], found the relation of root-mass to aerial part was 130 : 100 at beginning of bloom, but 218 : 100 at ripe-seed stage.

Thornton and Nicol made no attempt to determine the maximum number of cuts that might be taken. It should be borne in mind that the experiment was carried out under greenhouse conditions, which do not represent field conditions in our climate. Two clippings were made, and a third and later one might have been taken, if the experiment had been prolonged, but it was evident from the counts of nodules that by the end of August the plants were on the decline. This is not necessarily true under field conditions at that time of year, but the greenhouse conditions accelerated growth, and possibly August in the greenhouse might represent September in the field, so far as the age of the plants was concerned. At the beginning of the experiment, the greenhouse conditions were more favourable to growth than English field conditions. Hence in the experiment the lucerne could have been clipped twice with advantage to the yield of tops (though not the yield of root). Bearing in mind the difference in conditions, this experiment may be claimed to support the recommendation to cut spring-sown first-year lucerne only once. Even should the luxuriance of leafage of first-year lucerne tempt the grower to cut it twice, he should think of the check sustained by the roots when cut more than once, or too early in the season.

The experiment showed that the first-year lucerne clipped twice had at the end of August a slightly smaller weight of root than had unclipped lucerne a month earlier. In view of the results of this experiment, and in the light of remarks made in the preceding paragraph, it would seem

that under English farming conditions, spring-sown first-year lucerne, if cut once about August, would yield a large bulk of nutritious fodder without the plants' root-development receiving an undue check. There seems to be little need to fear that the lucerne in the field would become too stemmy if left uncut till fairly late in its first year. If cut too early, some bulk of forage would be lost and an early check would be given to root-development; the leaves of subsequent growth would fall off towards winter and be returned to the soil, but they would be more valuable as forage than as a top-dressing. Moreover, by later cutting the farmer would ensure a considerable gain in bulk of roots, which would have a more economic residual manurial value than would the leaves as top-dressing.

The basis of these conclusions has been broadened by the observations of other authors, who studied the effect of clipping or cutting lucerne in the field in its year of sowing. The valuable observations of McKee [6] have been quoted by Thornton and Nicol [4] and need not be given afresh here. McKee stated that he repeated in one year the work of a previous year on seedling lucerne, from which one deduces, without being quite certain, that the lucerne was sown afresh each year. He added a brief discussion of the effect of cutting upon yields in subsequent years. His experiments were made at Chico, California.

Sundelin and Franck [7] concluded from experiments in Sweden that lucerne should not be cut during the year of sowing. They sowed lucerne in mid-June in successive years and examined the effects upon the yield of two cuts in following years, of not cutting in the year of sowing, and of cutting about 61, 83, and 100 days after sowing. Marked depressions in yield followed after the later cuts, the effects, however, varying with season. Early cuttings in the year of sowing were made in August, and so enabled the plants to recover before winter. From the results of Sundelin and Franck it appears that the effects of cutting very young plants were largely offset when the plants were allowed to recuperate before the onset of winter. (Cf. Grandfield's results (Section III) on one-year-old plants.) In 1922 late autumn-cutting of newly sown lucerne prejudiced the yield for three years afterwards. In the case of lucerne sown in 1923, the cut whenever taken in that year severely depressed the yields of 1924 and 1925. The work of Sundelin and Franck further suggests the importance in Great Britain of sowing in spring if a cutting of economic size is desired in the year of sowing. Their plants cut in August, 61 days after sowing, were only 35-40 cm. high and had not reached the stage of beginning to bloom.

Two earlier observers recognized the harmful effects of cutting lucerne too harshly in its seedling year. Especially is careful management needful when it is wished to obtain a stand on types of land not thought to be particularly suitable for lucerne—as, for example, when lucerne is to be grown as a fire-break (Greenwood [8], Nicol [9]). In a short but valuable paper 'On the Growth of Lucerne on Thin Light Soils and Chalky Loams', Clayden [10], of Littlebury, suggested sowing lucerne (on such soils) 'about the second week in May *without any corn*. . . . If the season is favourable, the field will give a nice cut for the scythe at the end of

August or beginning of September, or else it may be fed with sheep or cattle; but it should not be depastured too closely.' Clayden added that on the soils of which he wrote lucerne would not yield its full crop to cutting until the third season. B[artholomew] Rocque, an eighteenth-century farmer at Walham Green, near London, wrote in the first edition of his book on lucerne [11]:

'In Languedoc they sow no Corn with it, and, when the Lucern is six or seven Inches high, they mow it; by which all the Seeds of annual Weeds are cut off and killed.'

The notion that early cutting of young lucerne somehow encouraged its vitality, and also had the beneficial effect of a fallow for suppressing weeds, persisted in the United States until the early years of the present century (for some quotations of typical opinions on these points, see McKee [6] and Graber *et al.* [12]). On very weedy ground, Cockayne [13] found that drastic cutting was the only practicable method of establishing a crop. Rocque, however, in the second edition [11] of his book, continued the above-quoted passage by the following:

'By my own Experience, I find they are in the wrong; for it bleeds it, so that the Plants make but little Progress when cut so young, and are a long time in recovering it. It ought never to be cut but when in Bloom.'

This is perspicacious, and suggests that Rocque made good use of the observations made in the four years which intervened between the editions of his book.

II. *The Effect of the Number of Times of Cutting Established Lucerne upon the Yields in Subsequent Years*

Hansen [14, 15] has published the results of two investigations on a field scale directed towards an examination of the effect of cutting lucerne a varying number of times in one year upon the crops in subsequent years. His work is little known and not easily available; also, it has not been well abstracted. Graphs summarizing some of his results obtained at Tystoft, Denmark [14] are reproduced below (Figs. 1 and 2). Hansen concluded that in established lucerne (not in its first year) three cuts per annum were necessary and sufficient. Unfortunately, his experiment gave no certain information upon the effects of cutting first-year lucerne, since his two fields were not comparably treated.

In Bornholm, Hansen [15] continued his continental Danish work by investigating the effects upon the following year's crop of cutting 2-year-old lucerne a varying number of times. A variety of treatments (inoculation, manuring, absence and presence of cover crop) were given upon 18 plots, but figures for the results of these treatments are not recorded. Each of the 18 plots was divided into four for cutting 1, 2, 3, and 4 times in one year (1908, 1910). In the following year (1909, 1911) each plot was cut three times. The results for a similar number of cuttings on each of the 18 plots appear to have been bulked, irrespective of manurial and other preliminary treatments. This is to be regretted, since data are scarce upon the effect of a cover crop upon a legume. The lucerne sown under a cover crop was not cut in the year of sowing, whilst the lucerne

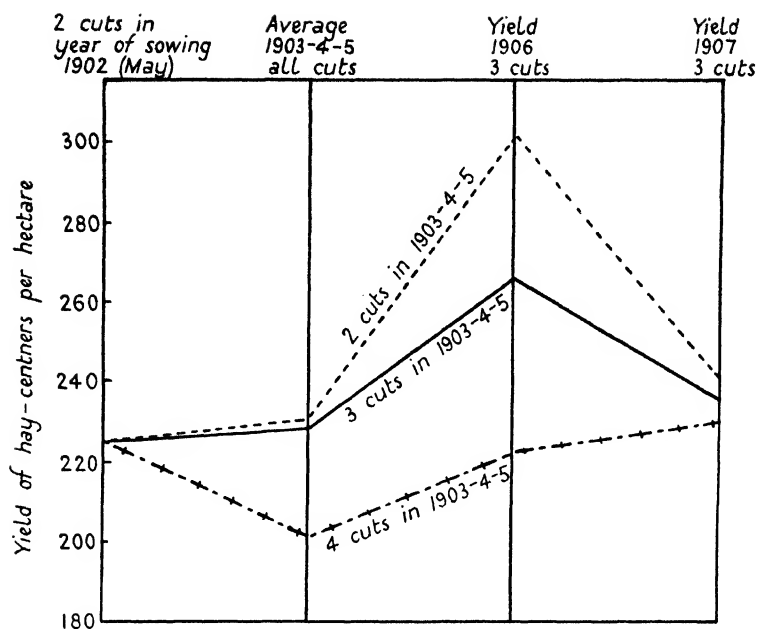


FIG. 1. Hansen's Tystoft data [14]. Varied number of mowings of spring-sown lucerne: yields in centners per hectare from Field I

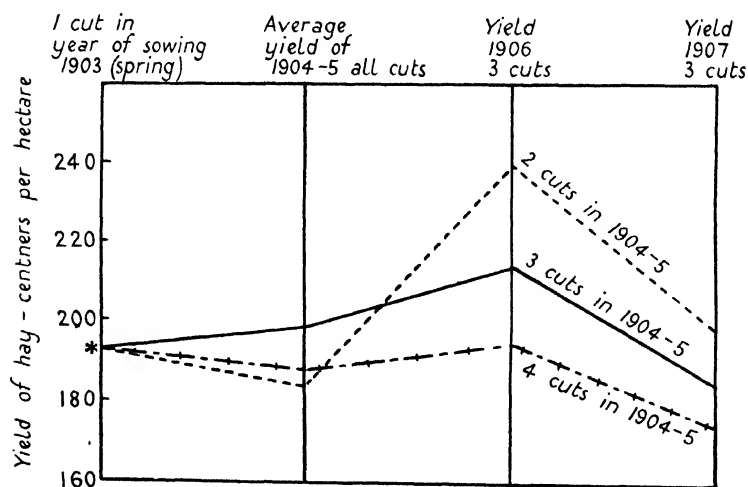


FIG. 2. Hansen's Tystoft data [14]. Varied number of mowings of spring-sown lucerne: yields in centners per hectare from Field II.

* This figure is not obtainable except from the corresponding graph in Hansen's paper, in the text of which it is stated that no weight for this cutting was recorded.

sown bare was cut once in September. Sowing was performed in May 1906. In 1907 all plots were cut twice (yields not recorded).

Hansen obtained his greatest yield over the period 1908-11, inclusive, by cutting once in 1908 and 1910 (8 cuts in all), but this result can be partly attributed to an unusually large yield in 1909. A steadier yield was given by cutting twice and three times in 1908 and 1910, whilst a marked reduction occurred throughout when four cuts were taken in those years. The striking fact emerges from his data that the total yield in each of the years 1908 and 1910 was hardly affected whether the lucerne was cut 1, 2, 3, or 4 times, but the reduction of yield in the following year increased with the frequency of cutting. It is probable that these reductions were really an expression of the adverse effects of late, rather than of too frequent cutting: Hansen's third cuts were taken in late August or late September, and his fourth cuts about October 10.

Hansen's results in Bornholm are summarized in Table 1.

TABLE 1

Data of Hansen's Experiments in Bornholm [15]

Effects of a varied and constant number of mowings in alternate years upon yields of 2-year-old spring-sown lucerne

A = number of cuts

B = annual yields of hay in centners per hectare

1908		1909		1910		1911		Total	
A	B	A	B	A	B	A	B	A	B
1	198	3	339	1	189	3	230	8	955
2	189	3	254	2	187	3	196	10	825
3	190	3	228	3	225	3	174	12	818
4	172	3	174	4	196	3	132	14	674

III. *Effects of Cutting upon Thickness of Stand*

Several authors [10, 16, 17, 38] have found that in established lucerne the yield of each of a small number of cuts in any one year falls off rather gradually as the season advances, though this does not appear to hold in some South American countries. This decrease in top-growth seems to be a physiological expression of the balance between root-reserves and photosynthesis, which is disturbed at each cutting. In the experiments of Hill and Thomas (18) defoliation was effected by fumigation with sulphur dioxide as well as by clipping. The clipping experiment of Thornton and Nicol [4], though made with seedling lucerne, by its observations upon roots throws some light upon the effects of such disturbances. The time separating the last cut of one year and the first cut of the next year is much longer than the interval between successive cuts in one season. A consideration of these facts suggests the great importance to lucerne of its winter-dormancy period. Neither in its seedling year nor in later years should the winter rest of lucerne be disturbed, if the best kind of stand is to be secured.

As already mentioned in the discussion of Hansen's results (see above),

it is necessary to separate the effects of lateness of cutting from those of frequency of cutting. Experiments on frequency of cutting have in some instances led to the taking of cuttings late in the year in order to crowd a number into one season. Amongst observations that have been made on the effects of late cutting of lucerne, those of Grandfield [19] may be quoted. Grandfield's findings confirm the recommendations of Moore and Graber [20]; his data are more specific than any others available, but his paper is difficult to abstract.

Grandfield's lucerne was sown in the autumn of 1928; his experiments began in the summer of 1929, and observations on the thickness of the stands were made until 1932 inclusive. A material loss of plants occurred where the plots were not protected through the winter. Protection of the plots (in reality, of the plants) was obtained by allowing the plants to remain uncut, so that the new growth after cutting was allowed to act as a mulch (as recommended by Thornton and Nicol [1]). The last cuts were made at various times, so that periods of 0 to 120 days elapsed before winter stopped growth. Three varieties of lucerne were tested, and, though these showed different degrees of winter-hardiness, the general conclusions were the same for all. No correlation was found between hardiness and liability to wilt disease. Grandfield recommended that lucerne should be allowed a growing period of 40 days after the last cutting; the length of the growing period before the onset of winter was a factor in maintaining the stands. Grandfield did not specify the location of his plots, but it may be assumed that the experiment was performed in Kansas. Albert [21], who examined more specifically the causes predisposing to winter-killing, concluded that 'the protection afforded to alfalfa plants by even small amounts of late-fall top-growth has a pronounced effect toward preventing winter-killing'. He also found that cutting the top-growth of plants at relatively immature stages retarded root-development.

Janssen [22] made some investigations upon varietal resistance to winter-killing. He gave weights of roots but did not clip the tops. The importance of his paper, from our point of view, lies in his demonstrations of the value of root-reserves and of the connexion between winter-killing and weed-invasion. Illustrations of badly winter-killed stands after late cutting were given by Moore and Graber [20]. Root-habit also plays a part in resistance to winter-killing (which is largely ascribable to the effects of alternate freezing and thawing, Southworth [23], Garver [24]), the branched roots of Grimm lucerne being particularly well adapted to resist heaving. Southworth has given several early references to the question of root-habit. For the effect of soil structure on root-systems of lucerne, see Carlson [25].

Salmon, Swanson, and McCampbell [26] in Kansas, working in 1914 with lucerne sown in 1912, made all their last cuts about the same time, thus eliminating the factor of late cutting studied by Grandfield. They found, in agreement with McKee [6] and Nelson [27], that early cutting reduced root-diameter. It also led to a reduction in the number of plants per plot (McKee) and to an increased invasion by grass. Nelson [27], who worked in 1921-3, inclusive, with lucerne sown in 1920 and left

undisturbed that year, did not publish absolute weights of lucerne roots but correlated weights with root-diameters; he also determined carbohydrate reserves as percentages. His presentation is neither uniformly made nor easy to follow, but it seems clear (if one of his minus figures is correctly interpreted as a gain) that the effect of clipping was to increase the soluble carbohydrate and to depress the amounts of other analysed constituents of the root. A review of the chemistry of reserves in lucerne has been made by Graber *et al.* [12], and by Leukel [28]—presumably the same as the Luekel of Graber *et al.* [12]. He concluded that lucerne cut frequently or late in the season in the succulent growth-stage goes into winter-dormancy low in organic root-reserves, and may suffer severe winter-injury. Albert [21] found that excessive cutting led to winter-killing, weed-invasion, and reduced life of crop, whether grass or lucerne: 'The initiation of new root-growth does not seem to occur to an appreciable extent until the rate of growth of the tops is relatively slow.'

The conclusions of the American workers just mentioned were strikingly anticipated by Graber [29] in a theoretical paper in which he pointed out that lucerne, by virtue of its clear-cut responses to cutting treatments, was a particularly valuable plant for investigations on root-reserves. Graber concluded that excessive or late cutting would lead to:

1. Smaller root reserves.
2. Lessened absorptive capacity.
3. Winter-killing.
4. Increased competition from weeds, due to thinning of the stand.

IV. *The Effects of Cutting Lucerne upon its Ability to resist Drought*

No record has been found of any experiment designed to examine drought-resistance in lucerne, but the foregoing discussion appears applicable to the problem of drought-resistance. Bates [30], who accords classical status to the work of Stapledon [31], wrote: 'It is a fact now generally accepted that the effect of denudation of foliage is to reduce the size and extent of the root-system.' This obviously has a bearing upon the drought-resistance of a normally deep-rooted plant such as lucerne. In fact, the demand which well-grown lucerne makes upon sub-soil water is so striking [32, 33, 34, 35] that it has been proposed to employ lucerne to lower the water-table under irrigated land [36].

Woodman, Evans, and Norman have recorded analyses of repeated cuttings made in 1-year-old and 2-year-old lucerne grown in Cambridgeshire. Their 1-year-old lucerne, sown at Howe Hill on light, gravelly soil in June 1932, was first mown in September 1932. The 2-year-old-stand was sown at Willingham on a deeper and heavier soil in June 1931 and cut twice in 1932. It is not recorded in their papers [37, 38] whether the Willingham lucerne was mown in 1931. A 'one-year-old stand', as that term is used by Woodman and his co-workers, is not the same thing as first-year, spring-sown lucerne, that is, lucerne sown about April.

Woodman and co-workers noted [38] that the 1-year-old lucerne at Howe Hill suffered severely during the drought of 1933; and claimed that this showed that light and fairly dry soils must be deep and not

easily dried out if lucerne is to maintain its reputation for enduring droughts. Whilst the shallowness of the soil may have been a factor contributing to the severity with which the lucerne at Howe Hill suffered from the dry weather in 1933, it is probable that the lateness of sowing (June) and the September cutting in the first year materially prejudiced the ability of the plants to withstand a drought in the following year. It is important to stress the effect of cutting the 3-months-old lucerne in 1932 upon the root-development in 1933. Given an adequate root-system, the lucerne plants at Howe Hill would probably have been able to withstand the 1933 drought even in a light, gravelly soil. Woodman *et al.* claimed that the results in 1933 have a special interest, in that they afford an insight into the influence of a prolonged drought on the yield, feeding-value, and general well-being of the crop. At least as far as yield and general well-being of the crop are concerned, this claim is of little value unless the effects of the previous year's treatment (June sowing and September mowing) are adequately considered. Cutting the tops dwarfs the roots, and, in Bates's words [30]: 'It is obvious that a restricted root-range will entail a limited water-supply.'

These considerations suggest that where lucerne is sown on gravelly soils, it should be sown about April if a cutting is desired in the year of sowing. If lucerne is sown in summer on soils liable to dry out, it should not be cut in the year of sowing if drought-resistance and a long duration of stand are desired.

V (a). *Effects of Clipping Clovers, Grasses, and Non-Legumes*

Lucerne is not peculiar in its root-response to defoliation. The effect, upon roots, over two seasons, of cutting the tops of grasses has been well studied by Stapledon [31], whose work, however, does not appear to be so well known to American agronomists as it should be. Other workers who may be mentioned here are Army [39], Paterson [40], Richardson *et al.* [41], Robertson [42], Parker and Sampson [43], and McCarty [44]. Sinclair [45] indeed in 1824 wrote: 'Nothing retards the after-growth of grasses so much as close cropping their first shoots, early in the spring.' His use of the term 'grass' included the clovers. Albert [21] found with two grasses that removal of the autumn growth appreciably lessened the amount of top-growth produced in the following season. Stewart [46] found that sweet clover (*Melilotus*) when closely cut produced little or no second growth. Bates' [30] work on *Trifolium* has already been mentioned. Voelcker showed in an interesting paper [47] that close grazing of clover led to a diminution in the residual manurial value to following wheat. Pierre and Bertram [48] working with the legume, kudzu, found its response to cutting to be closely similar to that of lucerne.

(b) *The Early Bite on Grassland*

The investigations here described undoubtedly have a bearing upon a dilemma which is experienced every spring by many farmers and graziers in Great Britain, even when their grassland does not contain lucerne; that is, the problem of the 'early bite'. There is a considerable temptation to turn stock on to the pastures and meadows in the early

spring, especially if stocks of hay and roots are short. Access to young grass is good for the stock, but it is always a question how far the utilization of the 'early bite' is economic. How far does grazing early in the year retard the development of grass and thus affect the ultimate yield from the grassland?

REFERENCES

1. H. G. THORNTON and HUGH NICOL, The Culture of Lucerne in Great Britain. J. Roy. Agric. Soc. Eng., 1932, **93**, 91.
2. H. E. WOODMAN, The Composition and Feeding-value of Lucerne. J. Min. Agric., 1934, **41**, 137.
3. H. G. THORNTON, The 'Inoculation' of Lucerne (*Medicago sativa*, L.) in Great Britain. J. Agric. Sci., 1929, **19**, 48.
4. H. G. THORNTON and HUGH NICOL, Some Effects of Clipping the Tops upon the Root-development of Lucerne. J. Agric. Sci., 1934. (In the press.)
5. G. WALTHER, Erprobtes vom Luzernbau. Die Phosphorsäure, 1934, **4**, 240.
6. R. MCKEE, The Effect of Clipping upon the Root-development of Alfalfa. J. Amer. Soc. Agron., 1916, **8**, 329.
7. G. SUNDELIN and O. FRANCK, Lucerneförsöken på Experimentalfältet. Medd. No. 335 f. Centralanstalten f. försöksv. på jordbruksområdet, Stockholm, 1928. (Swedish, with English summary.)
8. F. W. GREENWOOD, Lucerne as a Protection against Fire. New Zealand J. Agric., 1924, **28**, 103.
9. HUGH NICOL, Fires adjoining Railway Lines: a Useful Preventive. Modern Transport, 1934, **31**, No. 793, p. 9.
10. J. CLAYDEN, J. Roy. Agric. Soc. Eng., 1865, p. 359.
11. B. ROCQUE, A Practical Treatise on Cultivating Lucern Grass. London: 1761 and 1765.
12. L. F. GRABER, N. T. NELSON, W. A. LUEKEL, and W. B. ALBERT, Organic Food Reserves in Relation to Growth of Alfalfa and other Perennial Herbage Plants. Wisconsin Agric. Expt. Stat., Research Bull. No. 80, 1927.
13. A. H. COCKAYNE, Lucerne and Weeds. New Zealand J. Agric., 1915, **11**, 53.
14. P. HANSEN, Udbytteforsøg med Lucerne ved Afhugning et forskelligt Antal Gange i Sommerens Løb. Tidsskr. Landbr. Planteavl, 1909, **16**, 413. (Danish.)
15. — Forsøg med Afhugning af Lucerne paa Bornholm. Tidsskr. Landbr. Planteavl, 1912, **19**, 412. (Danish.)
16. J. G. OVERPECK and W. T. CONWAY, Alfalfa Variety Tests. New Mex. Agric. Expt. Stat. Bull. No. 152, 1926.
17. H. W. TURPIN, Lucerne Yields from Varying Irrigation Treatments. Farming in S. Africa, 1933, **8**, 384.
18. G. R. HILL and M. D. THOMAS, Influence of Leaf-destruction by Sulphur Dioxide and by Clipping on Yield of Alfalfa. Plant Physiology, 1933, **8**, 223.
19. C. O. GRANDFIELD, The Effect of the Time of Cutting and of Winter Protection on the Reduction of Stands in Kansas Common, Grimm and Turkestan Alfalfa. J. Amer. Soc. Agron., 1934, **26**, 179.
20. R. A. MOORE and L. F. GRABER, Wisc. Agric. Expt. Stat. Bull. No. 308, 1919; *ibid.*, 349. See also Annual Rept. for 1921-2, Bull. No. 352, 1923.
21. W. B. ALBERT, Studies on the Growth of Alfalfa and some Perennial Grasses. J. Amer. Soc. Agron., 1927, **19**, 624.
22. G. JANSSEN, The Relationship of Organic Root-reserves and other factors to the Permanency of Alfalfa-stands. J. Amer. Soc. Agron., 1929, **21**, 895.
23. W. SOUTHWORTH, A Study of the Influence of the Root-system in promoting Hardiness in Alfalfa. Sci. Agric., 1921, **1**, 5.
24. S. GARVER, Alfalfa Root Studies. U.S. Dept. Agric., Bull. No. 1087, 1922.
25. F. A. CARLSON, The Effect of Soil Structure on the Character of Alfalfa Root-systems. J. Amer. Soc. Agron., 1925, **17**, 336.
26. S. C. SALMON, C. O. SWANSON, and C. W. McCAMPBELL, Experiments relating to the Time of cutting Alfalfa. Kansas Agric. Expt. Stat., Tech. Bull. No. 15, 1925.

27. N. T. NELSON, The Effects of Frequent Cutting on . . . Alfalfa. J. Amer. Soc. Agron., 1925, **17**, 100.
28. W. A. LEUKEL, Deposition and Utilization of Reserve-foods in Alfalfa Plants. J. Amer. Soc. Agron., 1927, **19**, 596.
29. L. F. GRABER, J. Amer. Soc. Agron., 1924, **16**, 169.
30. G. H. BATES, The Relation of Leaf-size to Root-structure in *Trifolium repens*. J. Ecology, 1934, **22**, 271.
31. R. G. STAPLEDON, Seasonal Productivity of Herbage Grasses. Welsh Plant Breeding Sta., Bull. (Ser. H) No. 3, 1924, p. 5.
32. W. M. JARDINE and L. E. CALL, Alfalfa in Kansas. Kansas Agric. Expt. Stat., Bull. No. 197, 1914.
33. F. L. DULEY, The Effect of Alfalfa on Soil Moisture. J. Amer. Soc. Agron., 1929, **21**, 224.
34. T. L. KIESSELBACH, J. C. RUSSEL, and ARTHUR ANDERSON, The Significance of Subsoil Moisture in Alfalfa Production. J. Amer. Soc. Agron., 1929, **21**, 241.
35. T. A. KIESSELBACH, ARTHUR ANDERSON, and J. C. RUSSEL, Soil Moisture and Crop-sequence in relation to Alfalfa Production. J. Amer. Soc. Agron., 1934, **26**, 422.
36. E. S. WEST, Austral. Council Sci. Ind. Res., Bull. No. 74, 1933.
37. H. E. WOODMAN, R. E. EVANS, and D. B. NORMAN, J. Agric. Sci., 1933, **23**, 419.
38. ———, ibid., 1934, **24**, 283.
39. A. C. ARNY, Variations in the Organic Reserves in Underground Parts of Five Perennial Weeds. Univ. Minn. Agric. Expt. Stat., Tech. Bull. No. 84, 1932.
40. D. D. PATERSON, The Influence of Time of Cutting on the Growth, Yield, and Composition of Tropical Fodder Grasses. I. Elephant grass (*Pennisetum purpureum*). J. Agric. Sci., 1933, **23**, 615.
41. A. E. V. RICHARDSON, H. C. TRUMBLE, and R. E. SHAPTER, The Influence of Growth-stage and Frequency of Cutting on the Yield and Composition of a Perennial Grass—*Phalaris tuberosa*. Austral. Council Sci. Ind. Res., Bull. No. 66, 1932.
42. J. H. ROBERTSON, Effect of Frequent Clipping on the Development of Certain Grass Seedlings. Plant Physiol., 1933, **8**, 425.
43. K. W. PARKER and A. W. SAMPSON, Influence of Leafage-removal on Anatomical Structure of Roots of *Stipa pulchra* and *Bromus hordeaceus*. Plant Physiol., 1930, **5**, 543.
44. E. C. MCCARTY, Univ. California Thesis, 1927. Quoted by Parker and Sampson [43] as in the press, but not traced.
45. G. SINCLAIR, *Hortus Gramineus Woburnensis*. London: 1824 and 1826.
46. G. STEWART, J. Amer. Soc. Agron., 1934, **26**, 248.
47. A. VOELCKER, On the Causes of the Benefits of Clover as a Preparatory Crop for Wheat, J. Roy. Agric. Soc. Eng., 1868, p. 397. See also (in discussion) ibid., 1864, p. 537.
48. W. H. PIERRE and F. E. BERTRAM, Kudzu Production with special reference to Influence of Frequency of Cutting on Yields and Formation of Root Reserves. J. Amer. Soc. Agron., 1929, **21**, 1079.

(Received June 13, 1934)

THE EFFECT OF ENVIRONMENTAL CONDITIONS UPON PYRETHRUM (*CHRYSANTHEMUM CINERARIAEFOLIUM*). I.

By J. T. MARTIN,¹ Ph.D., A.I.C.

AND

F. TATTERSFIELD, D.Sc., F.I.C.

(*Department of Insecticides and Fungicides, Rothamsted
Experimental Station, Harpenden, Herts.*)

(With Plate XXXIII.)

CONTENTS.

	PAGE
Introduction	670
The effect of soil fertility	671
Fertiliser experiment, carried out in large pots	671
Sand-culture experiment	675
The pyrethrin content of the flowers from year to year	676
Distribution of the pyrethrins in the different parts of the flowers	678
Discussion of results	679
Summary	681
References	681

INTRODUCTION.

THE increase in the demand for insecticidal pyrethrum flowers has led to attempts to establish new centres of production. At present, most of the world supply of the flowers comes from Japan and Dalmatia, but the plant is cultivated on a smaller scale in France, Italy, North Africa and in Kenya Colony. Experimental cultivation of the crop is also in hand in many other countries and it has been shown that the plant will thrive in the temperate climate of England, and will produce good yields of flowers with a high poison content. Little is known, however, of the effect of soil or other environmental conditions upon the growth of the plant and the production of flowers, or of the factors which influence the poison content of the flowers. The possibilities of increasing the yield and toxic value of the flowers, and of prolonging the life of the plant by the application of manures are of economic importance.

¹ Being part of a thesis approved by the University of London for the Degree of Doctor of Philosophy.

The plant is a perennial and has been successfully cultivated on poor land. In Japan it is the custom to apply manure to the beds before the seeds are sown, and again at later stages in the life of the seedling, but the excessive use of a nitrogenous fertiliser is said to result in increased vegetative growth, at the expense of flower production. The plant there is grown on the hill slopes, and withstands severe winter conditions. Gnadinger, Evans and Corl(3) have shown that in Colorado little loss results from subjecting the plant to winter temperatures as low as -39°C . It is known that pyrethrum will thrive in a warm dry climate, and it is said to prefer a light calcareous soil, while wet, acid or heavy soil conditions have been regarded as unfavourable.

The elucidation of the chemical nature of the active principles of the flowers by Staudinger and Ruzicka(6), followed by the elaboration of chemical methods of assessing the toxicity of the flowers by Gnadinger and Corl(1) and Tattersfield and his co-workers(8), has made possible the investigation of the effect of soil and other conditions upon the insecticidal value of the flowers. The present work has been carried out with the object of defining more accurately the soil conditions under which the plant will flourish for a number of years and produce good yields of flowers of high quality.

Tattersfield (*loc. cit.*) has stated that there is a fairly close correlation between the pyrethrin I content and the toxicity of pyrethrum, and this has been confirmed by other investigators(5, 9) and substantiated by us in recent work. The relative activities of the flowers from plants grown in heavy soil under different manurial treatments have, therefore, been assessed by the determination of pyrethrin I. A more extended experiment using the field plot technique on light sandy soil is in progress, and will be described later.

THE EFFECT OF SOIL FERTILITY.

Fertiliser experiment, carried out in large pots.

This was commenced in 1929, using heavy soil from Broadbalk Field, Rothamsted, which had not been manured for many years. The soil used, which was neutral in reaction, gave the following figures on analysis, expressed on dry matter:

Carbon	1.85 per cent.
Nitrogen	0.17 ,,
Potash as K_2O	0.37 ,,
Phosphate as P_2O_5	0.14 ,,
Potash soluble in citric acid	0.012 ,,
Phosphate soluble in citric acid	0.012 ,,

The soil was mixed with 10 per cent. of sand, and weighed amounts of the fertilisers incorporated. The manurial treatments used were:

No manure.		
Farmyard manure.		
Complete dressing of artificial fertilisers.		
Complete artificials, excluding potash.		
„	„	nitrogen.
„	„	phosphate.
„	„	chalk.

Eighteen replicates were taken for the unmanured series, twelve replicates each for the farmyard manure and the complete artificial fertiliser treatments, and six replicates each for the nitrogen, potash, phosphate and chalk deficiency treatments.

The complete dressing of artificial fertilisers used per pot of 44 lb. of soil consisted of:

Potassium sulphate	5 gm.
Sodium nitrate	10 „
Superphosphate	10 „
Calcium carbonate	30 „

The farmyard manure was applied at the rate of 400 gm. of fresh manure per pot, equivalent to 20 tons per acre, while the sodium nitrate was added as fractional dressings in aqueous solution. A small amount of manganese (0.5 gm. per pot) was added (as sulphate) in most cases, but the soil contained sufficient of this element to preclude the possibility of deficiency.

The plots were plunged in a raised bed at the Plant Pathological Laboratory of the Ministry of Agriculture, and the whole series was planted early in June 1929 with rooted shoots from a single large plant of vigorous growth, thus avoiding the variable factors that would have been introduced if seedlings had been used. The pots were placed 18 in. apart, the positions in the bed being randomised. The plants were provided and the cultural work undertaken by the staff of the Ministry's Laboratory. A first harvest of flowers was obtained in 1930.

1930 harvest. The flowers from each plant were taken on one day, the majority being in the fully open condition. The stalks were completely removed, the heads dried in the shade in a warm glasshouse, and the yield from each plant obtained. The pyrethrin I percentage of the flowers from each plant was then determined.

1931 *harvest*. A separate investigation had shown that the maximum pyrethrin content occurs when the flowers are in the fully open condition. In harvesting the 1931 crop, the flowers were therefore taken from each plant as they came to maturity. A flower showing at least the first peripheral circle of disc florets completely open was regarded as a fully open flower. No differences, however, were seen in the time or rate of maturation of the flowers from the plants subjected to the various manurial treatments. The pyrethrin I content of the flowers from each plant was determined as before.

1932 *harvest*. The flowers from each plant were harvested when fully open, dried and weighed, and a representative sample taken for each manurial treatment for the determination of pyrethrin I.

From the weights of flowers yielded by the individual plants, the mean value, with the standard deviation of the mean, was calculated for each treatment used. These figures, together with the mean values for pyrethrin I, with their calculated standard errors where possible, are given in Table I.

Table I.
Results of fertiliser experiment commenced in 1929.

Treatment	Weight of flowers per plant gm.	Average weight of flowers gm.	Pyrethrin I % of flowers
1930 harvest.			
No manure	18.18 \pm 2.32	0.128	0.96 \pm 0.022
Farmyard manure	24.53 \pm 2.56	0.135	1.01 \pm 0.023
Complete fertilisers	24.30 \pm 3.10	0.129	0.94 \pm 0.032
No potash	28.18 \pm 3.90	0.134	1.00 \pm 0.010
No nitrogen	21.81 \pm 4.04	0.134	1.01 \pm 0.021
No phosphate	19.84 \pm 5.32	0.127	0.96 \pm 0.017
No chalk	18.05 \pm 3.00	0.140	1.03 \pm 0.028
1931 harvest.			
No manure	34.96 \pm 2.41	0.118	0.96 \pm 0.031
Farmyard manure	35.31 \pm 4.31	0.121	0.96 \pm 0.019
Complete fertilisers	35.87 \pm 3.40	0.118	0.99 \pm 0.014
No potash	45.02 \pm 5.82	0.112	1.02 \pm 0.017
No nitrogen	27.98 \pm 5.73	0.114	0.97 \pm 0.009
No phosphate	41.41 \pm 5.69	0.115	1.01 \pm 0.013
No chalk	37.81 \pm 3.82	0.120	0.98 \pm 0.011
1932 harvest.			
No manure	17.23 \pm 1.75	0.134	0.88
Farmyard manure	15.94 \pm 2.34	0.132	0.87
Complete fertilisers	19.49 \pm 2.50	0.132	0.87
No potash	13.44 \pm 2.76	0.115	0.80
No nitrogen	19.29 \pm 1.09	0.131	0.84
No phosphate	20.65 \pm 2.69	0.127	0.86
No chalk	19.51 \pm 2.07	0.124	0.80

There were large differences in the weights of flowers yielded by individual plants, and these differences are reflected in the standard

errors recorded. For the first year of the experiment a statistically significant increase in the yield of flowers over the yield from the unmanured series was obtained from the treatment where nitrogen and phosphate were supplied with omission of potash. This increase in yield, however, was not maintained in the second and third years of the experiment, when the plants grown in the unmanured soil gave a mean yield of flowers not significantly different from the yields obtained from the plants grown with the application of fertilisers.

For the 1930 and 1931 harvests, the pyrethrin I content of the flowers from the majority of the plants remained remarkably constant and approximated to 1 per cent. of the flowers. There was no significant difference in the mean values obtained for the treatments used. The average figures for pyrethrin I in 1932 were slightly lower than those of the previous years, but were still of a high order.

From the beginning of the experiment seven plants out of the sixty-six appeared to be exceptional. These in 1930 and again in 1931 gave values for pyrethrin I of 0.5–0.6 per cent., the content given by the remainder of the plants being of the order of 1.0 per cent. of the flowers. The exceptional plants did not show the normal vigorous growth exhibited by the majority of the experimental plants, and died before the harvest of 1932. The relatively low pyrethrin I content of the flowers would seem to be associated with the lack of vitality of the plant. The plants giving low values for pyrethrin I were omitted in compiling the final tables of average figures for the fertiliser treatments. The experiment was not continued after 1932, since at this stage some of the plants showed signs of failure.

A second fertiliser experiment was commenced in 1930, using again the heavy Rothamsted soil, and precisely similar manurial treatments, manganese, however, being omitted. Ten replicates were taken for the unmanured series, and eight replicates each for the remaining treatments.

In this case, however, the parent plant selected gave rooted shoots yielding flowers of pyrethrin I content approximating to 0.5 per cent. Pyrethrin I determinations were made on the flowers from each plant in 1931, and a representative sample of flowers from each treatment was analysed in 1932. The mean values, together with the standard errors, calculated for the fertiliser treatments for the first two harvests are given in Table II.

As in the previous experiment, an initial increase in the yield of flowers that was just statistically significant was recorded where nitrogen

and phosphate were applied alone, but again this result was not shown in the second year of the experiment.

Table II.
Results of fertiliser experiment commenced in 1930.

Treatment	Weight of flowers per plant gm.	Average weight of flowers gm.	Pyrethrin I % of flowers
1931 harvest.			
No manure	27.59 \pm 2.59	0.170	0.39 \pm 0.015
Farmyard manure	29.18 \pm 3.44	0.167	0.40 \pm 0.013
Complete fertilisers	28.96 \pm 1.57	0.159	0.38 \pm 0.008
No potash	35.33 \pm 1.76	0.161	0.37 \pm 0.011
No nitrogen	27.19 \pm 1.82	0.167	0.39 \pm 0.011
No phosphate	31.75 \pm 2.42	0.162	0.40 \pm 0.008
No chalk	34.84 \pm 2.50	0.166	0.40 \pm 0.011
1932 harvest.			
No manure	17.03 \pm 2.19	0.183	0.58
Farmyard manure	16.35 \pm 1.68	0.170	0.50
Complete fertilisers	13.71 \pm 1.45	0.152	0.54
No potash	16.74 \pm 1.83	0.160	0.57
No nitrogen	15.43 \pm 3.92	0.163	0.58
No phosphate	16.84 \pm 2.20	0.160	0.58
No chalk	18.90 \pm 3.31	0.164	0.55

The mean figures for the pyrethrin I content of the flowers were slightly higher in 1932 than those obtained for the 1931 crop. In each year, however, the flowers from the plants grown in the unmanured soil were significantly as rich in pyrethrin I as those from the plants in the manured series. The results tend to show, as before, that the application of manure has but little effect upon the pyrethrin I content of the flowers. The experimental bed of plants as they appeared just before the 1931 harvest is shown in Plate XXXIII.

The climatic conditions during the experimental period may be briefly summarised. A warm dry period which prevailed from the time of planting until September 1929 was followed by a mild period in which the rainfall and sunshine totals were above the average figures. The year 1930 was one of normal weather conditions, with normal sunshine, and with the rainfall evenly distributed throughout the year. The feature of 1931 was the deficiency in sunshine hours, while the temperature was often below normal. The summer months were cold and wet. The early months of 1932 were cold and dry, followed by phenomenal rainfall in May, and a drought in June, accompanied by subnormal sunshine.

Sand-culture experiment.

The yields and pyrethrin I contents of the flowers from the plants grown in the unmanured series of the experiments just described were high, and suggest that the poor soil used was rich enough to meet the

manurial requirements of the plant. An experiment to test definite deficiency treatments was therefore carried out, rooted shoots from one parent plant being grown in silver sand in glazed pots to which known quantities of fertilisers were added in solution in water.

The amounts of nutrients supplied to the fully manured plants were as follows:

Sodium nitrate	2.0 gm. per pot
Disodium phosphate	0.56 „
Potassium sulphate	0.41 „
Calcium chloride	0.10 „
Magnesium sulphate	0.28 „
Trace of iron and manganese				

Other pyrethrum plants were grown under conditions of nitrogen, phosphate and potash deficiency by the addition, in turn, of one-fifth of the amounts of sodium nitrate, disodium phosphate, and potassium sulphate supplied to the fully manured plants. Four replicates were used in each treatment. The majority of the plants made good growth, and gave flowers of high average weight (0.09–0.19 gm.). The pyrethrin I contents of the flowers from each treatment were determined and are given in Table III.

Table III.

*Plants grown in sand with deficiencies of nitrogen,
phosphate and potash.*

Treatment	Pyrethrin I % of flowers
Fully manured	0.35
Nitrogen deficiency	0.35
Phosphate deficiency	0.33
Potash deficiency	0.34

It is evident that, as far as this experiment indicates, there is no effect of the fertiliser constituents upon the pyrethrin I content of the flowers.

THE PYRETHRIN CONTENT OF THE FLOWERS FROM YEAR TO YEAR.

Differences may occur in the type of flowers produced by plants of *Chrysanthemum cinerariaefolium*. The flowers of some plants bear short ray florets, and these also appear in the flowers resulting from rooted shoots from these plants. Certain plants have been observed to give flowers maturing at an earlier or later date than the majority of the experimental plants, and this relative earliness or lateness of flowering

is again exhibited in plants derived from these individuals by vegetative propagation.

Large variations were noticed in the percentage of pyrethrin I and of the total pyrethrins in the flowers from different plants. We have found, however, that a given plant has yielded flowers containing approximately the same level of percentage of pyrethrin I from year to year when observations were made for at least three successive years (see Table IV). This approximately constant degree of richness in pyrethrin I of the flowers of a given plant appeared to be independent of the number of flowers produced each year. The total pyrethrin contents of the fully open flowers from eight individual plants were determined for two successive harvests, and approximately constant values were obtained for each plant (see Table V). The determination of the total pyrethrins in the flowers from these plants was not carried out after the second year. We are therefore unable to say whether the pyrethrin II found in the flowers of a given plant remains of the same order from year to year, as is the case with the pyrethrin I.

Table IV.

Fluctuations in pyrethrin I percentage of flowers from year to year.

Plant	Pyrethrin I % of flowers			
	1929	1930	1931	1932
EM 1	0.32	0.30	0.28	—
2	0.45	0.52	0.55	—
3	0.51	0.60	0.55	—
4	0.29	0.27	0.16*	—
5	0.30	0.28	0.22*	—
6	0.40	0.42	0.55	—
G 4	1.00	1.06	0.80†	0.91†
G 9	0.64	0.67	0.56†	0.59†
F 11	1.16	1.10	0.92†	0.88†

* Flowers were overblown.

† Rooted shoots from parent plants. The flowers from rooted shoots from G 4 and F 11 in 1933 gave 0.86 and 1.00 per cent. of pyrethrin I respectively.

Table V.

Total pyrethrins percentage of the flowers for two successive years.

Plant	1929		1930	
	Pyrethrin I	Total pyrethrins	Pyrethrin I	Total pyrethrins
G 4	1.00	1.98	1.06	2.10
G 5	0.74	1.47	0.63	1.41
G 7	0.53	1.27	0.53	1.42
G 8	0.95	1.80	0.97	1.84
G 9	0.64	1.78	0.67	1.81
G 10	0.30	0.96	0.30	0.79
G 12	0.67	1.47	0.66	1.58
F 11	1.16	2.02	1.10	2.08

DISTRIBUTION OF THE PYRETHRINS IN THE DIFFERENT
PARTS OF THE FLOWERS.

Fully open flowers, grown in England from Japanese seed, and having at least one-quarter of the disc florets open and no parts missing, were dissected into the constituent fractions, petals, receptacles and involucre scales, ovaries and the remainder of the disc florets. The percentage weight of each fraction in the flowers was ascertained, and the total pyrethrin contents of the fractions were determined by the micro-reduction method (4). The percentage distribution of the pyrethrins in the different parts of the flowers was then calculated, and is shown in Table VI.

Table VI.

Distribution of the pyrethrins in fully open flowers.

	Composition of flowers	Total pyrethrins
	%	%
Petals	25.2	0.18
Receptacles and involucre scales	20.4	0.27
Disc florets excluding ovaries	31.4	0.48
Ovaries	23.0	4.54

The sample from which the flowers were taken gave on analysis a total pyrethrin content of 1.37 per cent. The total pyrethrin content calculated from the figures obtained by the analysis of the constituent fractions was 1.30 per cent.

The ovaries were found to be much richer in pyrethrins than the remaining parts of the flowers. Of the total pyrethrins found in the fully open flowers examined, 80 per cent. was located in the ovaries, and about 10 per cent. in the remainder of the disc florets. Thus the disc florets of the flowers, made up of the corolla and stamens with the attached inferior ovaries, contained 90 per cent. of the pyrethrins found in the flowers.

The percentage of pyrethrin I in the ovaries taken from flowers that had just reached the fully open condition was determined. A value of 1.69 per cent. was obtained, while the average weight of the ovaries was 0.21 mg. The ripe fruits from the overblown heads, however, gave a value for pyrethrin I of 0.38 per cent., while the average weight of the seeds had increased to 0.80 mg. During the ripening of the ovaries there is, therefore, a fourfold increase in weight, while the poison content falls proportionately. This result confirms earlier work (7), when it was shown that the *percentage* pyrethrin content of the flowers falls after fertilisation

due to increase in the weight of the heads without a significant change in the absolute amounts of the pyrethrins present in the flowers.

DISCUSSION OF RESULTS.

An increase in the weight of the heads that was just significant was obtained in the first year of each manurial experiment from the treatment where nitrogen and phosphate were applied alone, but this result is not of practical importance. After the first year of the experiments, there were no significant differences in the yields of flowers from the plants grown in the unmanured and enriched soils. No differences due to the manurial treatments used were observed in the time or rate of maturation of the flowers.

At no stage in the experiments was a significant difference found in the pyrethrin I content of the flowers from plants in the unmanured series and in the fertilised series. In the sand-culture experiment, the plants grown under conditions of nitrogen, phosphate and potash deficiency gave flowers of pyrethrin I content equal to that of the flowers from the fully manured plants.

The data obtained show that when pyrethrum is grown on heavy soil under pot-culture conditions, the pyrethrin I content of the flowers is not influenced to any great extent by the application of manures. It appears that the plant will grow quite well on poor soil, and will produce flowers having a high poison content. In this connection, it is of interest that an experimental plot of pyrethrum at Harpenden, laid down in 1926 upon heavy soil to which no manure had been applied, has yielded rich flowers each year since 1927. A good crop was obtained in 1933, when the soil must have been in a low state of fertility.

It was hoped, as a result of the experiments carried out on the growth of pyrethrum in poor soil to which known amounts of fertilisers were added, to obtain information on the effect of soil fertility conditions upon the length of the life of the plant. The pot technique employed, however, proved unsatisfactory for this purpose, as, although the pots used were large, the root development of the plants was so considerable that the experiment could not be continued after the third year.

In order to determine the full effect of fertilisers upon the plant, particularly with regard to the number of years the plant will continue to give flowers, the yield and pyrethrin content of the flowers, an experiment is in progress on a poor sandy soil using the small plot technique, and will be reported upon later.

The data presented indicate that the extent to which the pyrethrins

are produced in the flowers is characteristic of a particular plant, and that it is not influenced to any extent by the enrichment of the soil.

Earlier work by Gnadinger and Corl(2) and Tattersfield(7) has shown that the pyrethrin content is at a maximum when the flowers are fully open. It is possible, however, that the flowers from one set of plants, at an early stage in their development, might prove richer than fully open flowers from another set of plants.

Although the genetics of pyrethrum are too complex to make any definite statement possible, the extent to which the pyrethrins are produced in the flowers appears to depend upon an inherent property of the plant in question. A plant initially producing flowers rich in pyrethrins has, with us, continued to produce flowers of a similar degree of richness for a number of years, and rooted shoots from the parent plant have again yielded rich flowers (see Tables IV and V, p. 677).

The average pyrethrin content of the flowers from a given bed of plants will depend upon the relative numbers in the bed of plants producing rich and poor flowers. A crop of flowers rich in pyrethrins might be secured by a careful selection of plants in the early stages of cultivation according to their virility and pyrethrin-producing power, or by the employment of cuttings from the roots of rich plants. We have not, however, up to the present time, been able to correlate the pyrethrin content of the flowers with any particular morphological characteristic of the plant, so that the process of plant selection, based upon chemical evaluation of the flowers of individual plants, is likely to be laborious. The number and size of flowers borne by a plant have an economic importance almost as great as the content of active principles, but whether there is a strict correlation between these factors is unknown.

The greater part of the pyrethrins of the fully open flowers are located in the ovaries. This confirms the result of Gnadinger and Corl(2). Nothing is known of the physiological rôle of the pyrethrins in the plant. The fact that there is no significant loss in their absolute amount after fertilisation suggests that they are not further metabolised in the flowers. We have no conclusive evidence to show that the pyrethrins are formed as waste products in the anabolic processes leading to the final maturation of the flowers, or that they play an important part in the subsequent metabolism of the seed.

SUMMARY.

1. The effect of soil fertility upon the insecticidal value of the flowers has been studied in a series of pot experiments. On heavy soil the pyrethrin I content of the flowers was not increased by the application of fertilisers. The plants produced good yields of flowers, rich in pyrethrins, when grown in soil of low fertility.

2. Under conditions of normal growth and vitality, the extent of production of the pyrethrins in the flowers was characteristic of the individual plant and was dependent upon some factor which appeared to be genetical in character. A plant initially producing flowers of high or low percentage poison content continued to give flowers of the corresponding degree of richness in succeeding years, independently of the application of manures, or apparently of the number of heads produced. Plants derived from rooted shoots produced flowers corresponding in quality with those initially yielded by the parent plant. The insecticidal value of pyrethrum flowers may be improved by plant selection, followed by vegetative propagation.

3. In the fully open flowers, the complete disc florets contained 90 per cent. of the total pyrethrins present in the flowers, and of this the greater part was found to be located in the ovaries.

We desire to express our most sincere thanks to Mr J. C. F. Fryer and Mr C. T. Gimingham of the Plant Pathology Department of the Ministry of Agriculture for the facilities placed at our disposal for carrying out these experiments, for the raising of the plant material used and for the care of the plants during the experiments.

REFERENCES.

- (1) GNADINGER, C. B. and CORL, C. S. (1929). *Journ. Amer. Chem. Soc.* LI, 3054.
- (2) ——— (1930). *Ibid.* LII, 680.
- (3) GNADINGER, C. B., EVANS, L. E. and CORL, C. S. (1933). *Colorado Exp. Sta. Bull.* No. 401.
- (4) MARTIN, J. T. and TATTERSFIELD, F. (1931). *Journ. Agric. Sci.* XXI, 115 (Rothamsted Memoirs, 15).
- (5) RICHARDSON, H. H. (1931). *Journ. Econ. Ent.* XXIV, 97.
- (6) STAUDINGER, H. and RUZICKA, L. (1924). *Helv. Chem. Act.* VII, 177.
- (7) TATTERSFIELD, F. (1931). *Ann. Appl. Biol.* XVIII, 602 (Rothamsted Memoirs, 17).
- (8) TATTERSFIELD, F., HOBSON, R. P. and GIMINGHAM, C. T. (1929). *Journ. Agric. Sci.* XIX, 266 (Rothamsted Memoirs, 14).
- (9) WILCOXON, F. and HARTZELL, A. (1933). *Contrib. Boyce Thompson Inst.* v, 115.

EXPLANATION OF PLATE XXXIII.

Fig. 1. Experimental bed of pyrethrum plants as they appeared just before the 1931 harvest.

(Received March 1st, 1934.)



MARTIN AND TATTERSFIELD.—THE EFFECT OF ENVIRONMENTAL CONDITIONS UPON PYRETHRUM
(*CHRYSANTHEMUM CINERARIAEFOLIUM*). I (pp. 670-681).

[FROM THE ANNALS OF APPLIED BIOLOGY, Vol. XXI, No. 4,
pp. 682-690, NOVEMBER, 1934.]
[All rights reserved.]

THE EFFECT OF ENVIRONMENTAL CONDITIONS UPON PYRETHRUM (*CHRYSANTHEMUM CINERARIAEFOLIUM*). II.

BY J. T. MARTIN¹, PH.D. A.I.C.

AND

F. TATTERSFIELD, D.Sc., F.I.C.

(*Department of Insecticides and Fungicides, Rothamsted
Experimental Station, Harpenden, Herts.*)

(With Plates XXXIV and XXXV.)

CONTENTS.

	PAGE
Introduction	682
Experimental	683
Discussion of results	688
Summary	689
References	689
Description of Plates XXXIV and XXXV	689

INTRODUCTION.

IN the preceding communication, the effect of soil fertility conditions upon the growth of pyrethrum (*Chrysanthemum cinerariaefolium*), and the pyrethrin content of the flowers was described. It was found that the plant will thrive on heavy neutral soil of low fertility, and that under the conditions of the experiment the pyrethrin I content of the flowers was not influenced by the application of manures. The factor governing the poison content of the flowers appeared to be genetical in character.

Other environmental conditions, however, are likely to affect the extent of production and the insecticidal value of the flowers. Of these, temperature, rainfall, the amount of sunlight, and the length of dormant period during the winter months appear to be the most important.

Pyrethrum will grow well and produce flowers of high insecticidal activity in regions of high altitude in Kenya Colony, although the flowering period is much extended, while in the lower regions, where a

¹ Being part of a thesis approved by the University of London for the Degree of Doctor of Philosophy.

higher temperature prevails, the morphological characters are changed and smaller yields of flowers are obtained. Moreover, in a strictly tropical belt below a certain altitude, where the plant continues to grow throughout the year, no flowers are produced. Gnadinger(1) reports that the experimental planting of pyrethrum in Kentucky was unsuccessful owing to the mild winter failing to induce a dormant condition, with subsequent injury due to sudden cold periods. The preclusion of sunlight during growth was shown by Gnadinger, Evans and Corl(2) to result in a reduced pyrethrin content of the flowers, but the flowers in this case were abnormal and small.

The present investigation had as its object the study of the effects of relatively high temperatures, partial shading, and of dormancy during the winter months upon the growth of pyrethrum and the production of flowers. Pyrethrin determinations have been made on the flowers from the experimental plants by the method of Tattersfield, Hobson and Gimingham(3).

EXPERIMENTAL.

In a preliminary experiment, fourteen rooted shoots of equal size and root development, from one parent plant, were grown in large pots in poor heavy soil, to which 10 per cent. of sand and a basal dressing of fertilisers were added. After 12 months' growth, the fourteen plants were randomised, and subjected to the following treatments:

Control plants. These were kept in the open under the normal climatic conditions prevailing over the experimental period.

Plants grown at a higher temperature. These were kept in a glasshouse heated during the winter months, and maintained at 15–25° C.

Partially shaded plants grown at the higher temperature. These were kept in the glasshouse as before, but were allowed half the normal amount of sunlight.

The shading of the plants was effected by means of beaver-board boxes darkened on the inside. The plants were covered and exposed alternately each day at noon. In this way, differences in the value of morning and afternoon sunlight were eliminated. The amounts of rain falling upon the control plants were recorded, and were taken into account in supplying water to the glasshouse plants.

The experimental treatments were commenced in February 1931. When near the time of flowering, some of the glasshouse plants were attacked by mites, but were successfully treated with colloidal sulphur. The flowers were taken as they reached the fully open condition.

684 *Effect of Environmental Conditions upon Pyrethrum*

The plants grown at 15–25° C. in full sunlight were the first to flower. The flowering period extended from the beginning of April until the beginning of June 1931, and showed a peak at the end of April. The shaded plants at 15–25° C. commenced to flower later, and flowered over a shorter period. The flowering of the control plants commenced at an even later date, the flowering period was short, and the yield of heads was greatly in excess of the yields from the glasshouse plants. There was a pronounced falling off in the average weight of the heads as flowering proceeded. The flowers were analysed for pyrethrin content by the acid method, or, where the weight of the flowers obtained was insufficient, by the micro-reduction method. The average figures for each treatment are given in Table I, and it will be seen that the differences between the treatments are sharply defined.

Table I.

The effect of summer temperature and shading upon pyrethrum.

	Control plants at 2–15° C.	Plants at 15–25° C.	Shaded plants at 15–25° C.
Flowering period in days	22	56	30
No. of flowers per plant	106	82	33
Weight of flowers per plant, gm.	14.37	5.91	1.29
Average weight of flowers, gm.	0.136	0.072	0.039
Pyrethrin I per cent. of flowers	0.42	0.36	—
Total pyrethrins per cent. of flowers	1.65	1.25	0.94*
Pyrethrins formed per plant, gm.	0.237	0.074	0.012

* By micro-reduction method.

The growth of the plants at a higher summer temperature than that to which the control plants were subjected is seen to result in a longer flowering period, and reduction in the number, average weight and pyrethrin content of the flowers formed. The effect of shading at the higher temperature is further to reduce the number of heads given, their average size and pyrethrin content. The differences in pyrethrin content of the flowers are clearly seen when the amounts of pyrethrins, in grams, formed per plant and located in the flowers are compared.

The glasshouse plants, from which the flower stalks and mature foliage were removed after the last of the experimental plants had flowered, were kept unshaded in the glasshouse at 15–25° C. through the winter of 1931–2. The plants continued to grow well, but no flowers were produced in the summer of 1932. Morphological changes were noticed: the leaves were originally small and deeply pinnatifid, but new leaves produced during growth at the higher temperature were much larger and less deeply pinnatifid. Three of the glasshouse plants, taken at the

normal time of flowering in June 1932 after continuous growth for twelve months, are seen in Plate XXXIV, fig. 1. The foliage was taken in July 1932, air-dried, and analysed for pyrethrin I. A value of 0.04 per cent. was obtained. Thus the foliage of the plants which produced no flowers had no insecticidal value.

A second experiment was commenced in 1931 in which the effect of shading the plant at normal temperatures and the effect of dormancy were studied. Twelve rooted shoots from each of two parent plants (A and B) were grown as before in large pots in heavy soil. Each set of twelve plants was divided at random into four groups of three plants each. Of these, two groups spent the winter of 1931-2 exposed to the normal climatic conditions, while the remainder were kept in the glasshouse at 15-25° C. Partial shading, carried out as in the previous experiment, of half the plants in the open and half the glasshouse plants was commenced in the spring of 1932. The addition of water to the glasshouse plants and the outside shaded plants was controlled in accordance with the amount of rain falling upon the unshaded plants.

The plants grown under the normal climatic conditions in both the unshaded and shaded series gave flowers, but as the plants were young the numbers of heads were small. The flowers from each plant were taken as they reached maturity, air-dried and weighed, and pyrethrin I determinations made. The plants in the glasshouse showed vigorous growth with the formation of large leaves, but with the exception of two plants, which formed a single small head each, no flowers were produced.

The average figures obtained for the plants from A and B grown under normal climatic conditions with and without partial shading are given in Table II. The chief effect of shading is seen to be the reduction in the average weight of the flowers, while the pyrethrin I content is also lowered.

Table II.

The effect of shading at normal summer temperature.

	Unshaded plants		Shaded plants	
	A	B	A	B
No. of flowers per plant	23	20	30	10
Weight of flowers per plant, gm.	4.92	4.66	3.29	1.64
Average weight of flowers, gm.	0.214	0.233	0.110	0.164
Pyrethrin I per cent. of flowers	0.48	—	0.43	—

The plants produced flowers to the normal extent only when they had been allowed a dormant period during the preceding winter. It was therefore decided to bring some of the outside plants into the glasshouse before the winter of 1932-3 and again in the spring of 1933, and

also to subject some of the glasshouse plants to the outside conditions, with the object of observing the effects of dormant and non-dormant conditions upon subsequent flower production. The foliage formed during the previous year was removed from all the plants and only the young foliage retained. The plants in the glasshouse and outside were divided at random into three groups of four, two plants in each group being derived from the parent plant A, and two from B. The outside plants were treated as follows: one set of four was taken into the glasshouse at the end of July 1932, a second set was removed inside during March 1933, and the remaining four plants were allowed to remain outside under the normal climatic conditions. Similarly, one set of four glasshouse plants was taken outside in July 1932, one set during March 1933, and the third set was retained in the glasshouse. The plants were allowed gradually to acclimatise themselves to their new conditions.

The plants which had spent 1931-2 under normal conditions and which had flowered in 1932 continued to grow on removal to the higher temperature conditions, and did not experience a dormant state during the winter of 1932-3. A few flowers were produced by each plant in June 1933. The effect of the higher temperature immediately before flowering is seen in the second set of outside plants which were moved into the glasshouse in the spring of 1933, after a winter dormant period. Here large numbers of flowers were produced, but the flowering was hastened, all the flowers being in the fully open condition early in May 1933. The control plants, *i.e.* those which had a dormant period in 1931-2, and again in 1932-3, and which were allowed to flower in 1933 under normal climatic conditions, gave in June rather fewer flowers than the preceding series, but the heads were of a higher average weight.

The four plants which had not experienced a dormant period during the winter of 1931-2 resulting in no flower production in 1932, and which were returned to the normal climatic conditions in July 1932, gave large numbers of flowers in 1933, apparently the effect of a dormant period during the preceding winter. On the other hand, the plants which had no dormant period either during 1931-2 or 1932-3, but which were allowed the normal summer conditions in 1933, gave no fully open flowers, but merely a few buttons, none of which reached maturity. The plants which had spent the whole of the experimental period at 15-25° C. gave a few fully open flowers in 1933, but this may have been due to the fact that they were situated in the glasshouse near a ventilator. The average numbers of fully open flowers that were obtained under these various conditions are given in Table III. The numbers of fully open

flowers given do not represent the full extent of the flower production, since flower-heads in the button to half-open condition were present. The average number of these left on each plant after the fully open flowers were taken is given in brackets.

Table III.

The effect of winter dormancy and different summer temperatures upon the flowering of pyrethrum.

Treatment	Series	Flowers per plant	Weight of flowers per plant gm.	Average weight of flowers gm.
Dormant 1931-2, non-dormant 1932-3, high temperature 1933	A	14	1.80	0.129
	B	19	2.13	0.112
Dormant 1931-2, dormant 1932-3, high temperature 1933	A	80	6.37	0.080
	B	105	9.26	0.088
Dormant 1931-2, dormant 1932-3, normal temperature 1933	A	67 (54)	7.12	0.106
	B	49 (97)	6.79	0.138
Non-dormant 1931-2, dormant 1932-3, normal temperature 1933	A	53 (75)	5.45	0.103
	B	52 (112)	6.36	0.122
Non-dormant 1931-2, non-dormant 1932-3, normal temperature 1933	A	(3) }	Gave no fully open flowers	
	B	(45) }		
Non-dormant 1931-2, non-dormant 1932-3, high temperature 1933	A	5	0.46	0.092
	B	11 (3)	1.04	0.094

Photographs were taken in May 1933, showing the early flowering of the plants which were subjected to the higher temperature after two dormant periods, and again in June 1933, at the normal time for the flowering of pyrethrum in this country (Plate XXXIV, figs. 2, 3 and Plate XXXV, figs. 4-6).

The differences in the appearance of the plants grown without a dormant period and those which, following a dormant period, produced flowers, are clearly seen in Plate XXXIV, fig. 2. Plant No. 2 had no dormant period during the winter of 1932-3, and showed large leaves, while No. 4, kept under the normal climatic conditions throughout the experiment, had small feathery leaves, the greater part of the plant being made up of thick flowering stems. The results indicate that under conditions which induce the continuous growth of the plant throughout the year, growth takes place at the expense of the production of flowers.

During the experimental period, the extremes of the temperatures recorded in the glasshouse were 11 and 30° C. The average monthly temperature in the glasshouse during the winter months fluctuated between 16 and 20° C., while during the summer average monthly temperatures of 20-24° C. were recorded. The lowest temperature that the plants under normal conditions experienced was -10° C. on one day

688 *Effect of Environmental Conditions upon Pyrethrum*

each in March 1932 and January 1933. The lowest average monthly temperature recorded (outside screen temperature) was 2° C. in February 1932, and the highest 16° C. in July 1932.

DISCUSSION OF RESULTS.

Gnadinger, Evans and Corl(2) have studied the effect of growing pyrethrum in full sunlight, partial sunlight, and in the absence of sunlight. When grown in partial sunlight, the numbers of flowers were reduced, while the average weight of the flowers was less than that of the flowers from the plants grown in full sunlight. The plants grown in partial sunlight actually gave flowers richer in pyrethrins than the control plants, but it was concluded that this was due to differences in the pyrethrin-producing power of the individuals used. We have endeavoured to eliminate the factor of individuality by using rooted shoots from a single plant. Gnadinger found that the growth of the plant in the absence of sunlight resulted in relatively few small flowers of low pyrethrin content. We have found that when pyrethrum is grown at a summer temperature of either 15 or 25° C. reduction in the amount of light available to the plant results in smaller flowers of poorer quality. The lowering of poison content may possibly be due to the less intense metabolism on reduction of the light, the plant showing under these conditions less vitality and growth. The illumination reaching the plants kept in the glasshouse would be deficient in light of short wave-length, as this would partially be eliminated in passage through the glass.

The effects of temperature and dormancy upon the production of flowers by pyrethrum are closely interrelated. The influence of a high temperature apparently depends upon the time at which it is in operation. The plants which, following a winter dormant period, were subjected to a high summer temperature, gave large numbers of flowers, but the flowering took place at an early date, while in no case did those which had been subjected to the higher temperature during the winter months produce more than a few flowers in the following summer. As far as our experiments show, the production of flowers in the summer is dependent upon dormancy in the preceding winter only, since the plants which, after active growth during the winter of 1931-2, were exposed to cold winter conditions in 1932-3, produced flowers in the summer of 1933. The type of foliage formed by the plants grown under the higher temperature conditions was associated with the inability to produce large numbers of flowers. The few flowers formed by these plants were attached to long, straggling and weak flowering stalks. The normal rapid flowering of

pyrethrum thus depends upon sufficient differences in the winter and summer temperatures. The winter temperature must be sufficiently low to induce a reasonably extended dormant period.

The results of these experiments have provided an indication that the pyrethrins are not formed photosynthetically in the leaves with subsequent translocation to the flowers. The foliage of the individuals grown at the higher temperature with non-production of flowers was not found to be richer in pyrethrins than that of the plants which flowered normally. It is reasonable to suppose that if the pyrethrins had been formed in the leaves, then in the absence of flowers the foliage would have been richer in pyrethrins than was actually found to be the case.

SUMMARY.

1. The effects of light, temperature and dormancy upon pyrethrum have been studied. The partial shading of the plant during the five months preceding flowering resulted in the production of smaller flowers with a reduced pyrethrin content.

2. The successful flowering of the plant was largely dependent upon the relative temperatures experienced throughout the year. A dormant period, dependent upon sufficiently low winter temperatures, was shown to be necessary for the normal production of large numbers of flowers.

REFERENCES.

- (1) GNADINGER, C. B. (1933). *Pyrethrum Flowers*. Minneapolis: McGil Lithograph Co.
- (2) GNADINGER, C. B., EVANS, L. E. and CORL, C. S. (1933). *Colorado Exp. Sta. Bull.* No. 401.
- (3) TATTERSFIELD, F., HOBSON, R. P. and GIMINGHAM, C. T. (1929). *Journ. Agric. Sci.* XIX, 266 (Rothamsted Memoirs, 14).

EXPLANATION OF PLATES XXXIV AND XXXV.

(Normal time of flowering is end of June.)

PLATE XXXIV.

Fig. 1. Taken June 24th, 1932. Plants at normal time of flowering after twelve months' continuous growth. No flowers formed.

Figs. 2, 3. Taken on May 11th, 1933. Plants 2, 14, dormant 1931-2, non-dormant 1932-3, high temperature 1933. Plants 9, 18, dormant 1931-2, dormant 1932-3, high temperature 1933. Plants 4, 24, dormant 1931-2, dormant 1932-3, normal temperature 1933. Flowers on 4 and 24 in button stage.

PLATE XXXV.

Fig. 4. Taken on May 11th, 1933. Plant 1, non-dormant 1931-2, non-dormant 1932-3, normal temperature 1933. Plant 5, non-dormant 1931-2, dormant 1932-3, normal temperature 1933. Plant 11, non-dormant 1931-2, non-dormant 1932-3, high temperature 1933. Flowers forming on 5.

Fig. 5. Taken June 22nd, 1933. Plants as in Fig. 4, plant 5 in flower, and few flowers on 11.

Fig. 6. Taken June 22nd, 1933. Plant 21, treatment given under 5 in Fig. 4. Plant 23, treatment given under 1 in Fig. 4. Plant 13, treatment given under 11 in Fig. 4. Few flowers shown on 23 and 13.

(Received March 1st, 1934.)



Fig. 1.

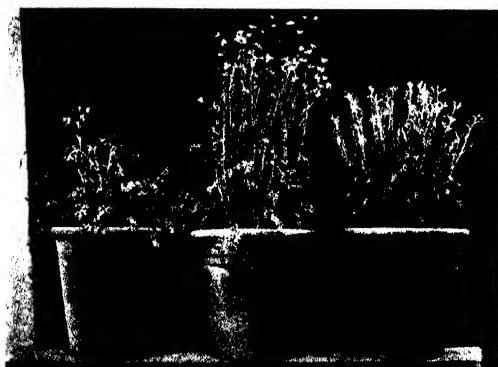


Fig. 2.



Fig. 3.

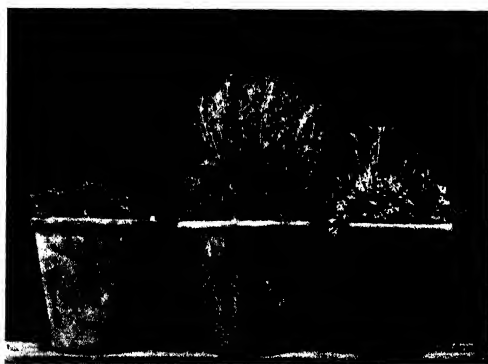


Fig. 4.



Fig. 5.



Fig. 6.

[FROM THE BIOCHEMICAL JOURNAL, VOL. XXVII, No. 3, pp. 818-831, 1933]

[*All Rights reserved*]

CVIII. A NEW METHOD FOR THE DETERMINATION OF CELLULOSE, BASED UPON OBSERVATIONS ON THE REMOVAL OF LIGNIN AND OTHER ENCRUSTING MATERIALS.

BY ARTHUR GEOFFREY NORMAN
AND SAMUEL HARRY JENKINS.

*From the Fermentation Department, Rothamsted Experimental Station,
Harpenden, Herts.*

(Received May 1st, 1933.)

IN the analysis of plant materials and woods, one of the chief constituents to be determined is cellulose, which is important since it forms the structural framework of the tissue. The determination of cellulose, however, is for various reasons not always satisfactory. The methods of determination of cellulose in general use do not all give the same product; consequently the results obtained are not always directly comparable. The method selected may be dictated by the purpose for which the cellulose is required. As it occurs naturally, cellulose is always found in the closest association with other constituents of the cell-wall, and these have, by various treatments, to be removed. Originally it was held that a number of distinct compound celluloses existed, *e.g.* lignocelluloses, pectocelluloses, *etc.* [Cross and Bevan, 1895], but the more recent view, which is generally accepted, is that all natural celluloses contain a certain fraction which is identical chemically. This fraction, typified by pure cotton cellulose, will from different sources give identical X-ray diagrams, comparable yields of cellobiose octa-acetate, and solely glucose on hydrolysis. It may therefore be regarded as "true" cellulose or "pure" cellulose in the rigid chemical sense. But cellulose as usually isolated does not yield only glucose on hydrolysis. In the case of hardwoods, cereal straws and certain fibre plants, xylose also is obtained, while in the case of softwoods mannose may be found in addition. These sugars are derived from polysaccharide material very intimately associated with the "pure" cellulose itself, and held either by adsorption or secondary valency forces. The association is so close that these polysaccharides may only be completely removed intact by the most violent treatment with alkali. Mild acid hydrolysis on the other hand removes them rapidly. These associated polysaccharides, which possess the general properties usually described for the hemicelluloses, have been termed by Hawley and Norman [1932] "cellulosans" and will be referred to as such in this paper. Development studies on the barley plant [Norman, 1933] show that the cellulosans are laid down with the "true" cellulose from the earliest stages and are not produced as the result of changes during lignification or senescence. The inference from this and other work is that the cellulosans must be regarded as forming a normal integral part of the cellulosic fabric of the plant, *i.e.* natural celluloses consist of "true" cellulose *plus* associated cellulosan. These two substances together will be taken in this paper to be covered by the word "cellulose."

As ordinarily found, natural cellulose is associated or encrusted with lignin and hemicelluloses of a polyuronide nature. Recent work on the molecular anatomy of "true" cellulose, as revealed by X-ray studies, makes it unlikely that primary valency linkages are involved in the association of lignin and cellulose, and physical theories of adsorption are usually proposed. Methods of cellulose determination therefore involve the removal of this lignin and encrusting hemicelluloses. The requirements of an ideal method are that lignin and polyuronide hemicelluloses should be rapidly and completely removed without affecting the integrity of the natural cellulose. It is in this last respect that a number of existing methods fail, inasmuch as they involve some treatment which attacks the less resistant cellulosan fraction.

The effect of pre-treatment on the yield of cellulose.

The first and by far the most important method is the chlorination process of Cross and Bevan, in which, by exposure to gaseous chlorine, a lignin-halogen derivative is formed and subsequently dissolved out together with the hemicelluloses by boiling with neutral sulphite. Alternate chlorination and boiling with sulphite have to be continued till, on addition of the sulphite, there is no red colour reaction indicative of lignin. In the case of many woods and fibres some three or four brief chlorinations are sufficient, but with the limited number of woods used in this work the number was considerably higher. Cross and Bevan [1895] originally proposed an alkaline pre-treatment of the material before chlorination, and boiled for 20 minutes with 1 % NaOH. Wood chemists in their official methods have, however, dispensed with this pre-treatment on the ground that the yield of cellulose is lowered thereby, as claimed by Renker [1910]. In the case of certain non-timber materials, as, for instance, cereal straws, chlorination without pre-treatment is an extremely tedious operation, in that as many as 9 or 10 exposures to chlorine and subsequent extractions with sulphite may be required, during which the cellulose becomes gelatinous and difficult to handle. In these cases an alkaline boiling is of great advantage, since the number of chlorinations then necessary is reduced to 2 or 3. Dorée [1933], discussing this point, advocates boiling for 5 minutes with 1 % NaOH on the grounds that it enormously facilitates subsequent delignification. It has been claimed that in the case of certain woods a lignin-free cellulose product is not attainable without prior alkaline treatment [cf. Strong, 1928]. Norman [1929], in work on the decomposition of cereal straws, employed the original Cross and Bevan procedure of boiling for 20 minutes with 1 % NaOH. There is little doubt, however, that advantageous as is this pre-treatment in expediting the removal of lignin, it is objectionable in that the cellulosan fraction may be partly attacked and suffer loss. Hawley and Norman [1932] drew attention to this on theoretical grounds and some experimental evidence on the point is given in Table I. Samples of three cereal straws were extracted for 20 minutes with boiling 1 % NaOH, filtered and washed thoroughly with hot water and a little dilute acetic acid. After drying and grinding, cellulose was determined by the Cross and Bevan method without alkaline pre-treatment. Total furfuraldehyde, lignin and furfuraldehyde due to cellulosan were also determined.

The effect of the alkaline pre-treatment on the cellulose yield is seen, as expected, to be a depressant one, the figures after treatment being less by the amount of cellulosan extracted. A measure of this is obtained from the figures for furfuraldehyde from the cellulose products, inasmuch as the cellulosan in the case of these three straws is a xylan. That subsequent delignification is

Table I. *Effect on cellulose determination of pre-treatment of straws with alkali.*
(Each straw boiled for 20 minutes with 1 % NaOH.)

All results expressed on the basis of 100 g. original dry straw.

Straw	Residue after extraction	Cellulose after extraction*	Furfurald. from cellulose after extraction	Cellulose on original straw	Furfurald. from cellulose on original straw	Apparent lignin on original straw	Apparent lignin after extraction	Total furfurald. on original straw	Total furfurald. after extraction	Non-cellulosic furfurald. on original straw	Non-cellulosic furfurald. after extraction
Oats	52.00	48.7	7.09	53.4	9.30	19.99	5.14 (74.30)	15.86	7.99 (49.60)	6.53	0.90 (86.20)
Wheat	62.52	53.7	9.55	55.2	9.96	20.93	6.02 (71.20)	16.64	10.58 (36.40)	6.68	1.03 (84.60)
Rye	66.31	57.2	10.18	60.2	11.44	21.56	6.97 (67.70)	14.80	12.05 (18.60)	3.36	1.87 (44.20)

* Cellulose determined by Cross and Bevan method without pre-treatment.

Figures in brackets represent percentage removed by pre-treatment with alkali.

facilitated is not surprising since the alkali treatment itself removes approximately 70 % of the total lignin of each straw. In addition a considerable proportion of the non-cellulosic furfuraldehyde-yielding constituents—the polyuronides—is removed by the pre-treatment.

Because of the difficulties mentioned above in determining cellulose in cereal straws by the Cross and Bevan procedure, Jenkins [1930] proposed a method involving the use of hypochlorite. Before submitting the straw to this reagent, however, it is boiled alternately for 5 minutes each with 1 % NaOH and 1 % HCl, these treatments being repeated twice. Jenkins [1930] quotes figures to show that the method gives results definitely lower than does Cross and Bevan's, but that this difference is due to loss of cellulosan rather than attack on "true" cellulose, which is identical in both cases. Unfortunately the figures were compared against the chlorination method with an alkaline pre-treatment. Had this been omitted, it would have been seen that the loss of cellulosan was great, and no doubt due to the hydrolysing effect of the acid boiling. To demonstrate in detail the losses occurring in each stage of the procedure advocated by Jenkins [1930] a large sample of straw was put through the process and analyses were made at each stage. By the Jenkins method the straw gave a "cellulose" figure of 39.56 %. The results are given in Table II.

Table II. *Analysis of effects of pre-treatments in Jenkins's cellulose method, using oat straw.*

All results expressed on basis of 100 g. original straw.

Treatment	Residue	Cellulose *	Furfurald. on cellulose	Total furfurald.	Non-cellulosic furfurald.	Apparent lignin †
None	—	53.5	9.2	15.9	6.7	19.9
Extracted twice with 1 % NaOH (5 mins.)	49.9	42.2 (21.2)	6.2 (33.2)	8.3	2.1 (68.7)	4.4 (77.9)
Extracted twice with 1 % HCl (5 mins.)	62.2	43.3 (19.2)	4.9 (47.2)	8.3	3.4 (49.2)	13.5 (32.2)
Extracted twice with alkali and acid (5 mins. each)	41.6	37.8 (29.3)	4.4 (51.9)	5.1	0.7 (89.5)	4.0 (79.9)

* By method described later in this paper.

† By 72 % sulphuric acid method.

Figures in brackets represent the percentage loss due to the treatment.

Although the individual treatments are brief the hydrolysing and extracting effects produced by them are very considerable. Alkali extracts more than does acid, mainly by reason of the extra lignin which it removes. The total furfuraldehyde yields of both acid- and alkali-extracted straw are identical but made up in rather a different way. The acid extraction apparently removes by hydrolysis over 45 % of the cellulosan, and alkali by solution less than 35 %, while on the other hand the effects of the two treatments on the non-cellulosic furfuraldehyde-yielding constituents are in the opposite direction. The apparent loss of lignin on brief treatment with acid was quite unexpected and will be referred to later. The combined effect of alkali and acid is a very drastic extraction of the straw, which loses nearly 70 % of its total furfuraldehyde-yielding groups and 80 % of its apparent lignin. The most serious result from the point of view of the subsequent determination of cellulose is that, by calculation from the furfuraldehyde in the cellulose, nearly 50 % of the cellulosan is removed. Pure cellulose, as typified by a high grade cotton, was shown by Jenkins [1930] to be unaffected by this treatment. The result of the subsequent three hypochlorite treatments is evidently very slight in comparison with that of the pre-treatments, for in this case the residue after the latter was 41.6 %, which was reduced to 39.56 % by the hypochlorite. Because of this slight effect of hypochlorite it was thought to be worth while to determine the lignin content of celluloses prepared by the Jenkins procedure. Some figures for straws are given in Table III, together with those for the Cross and Bevan product from the same material.

Table III. *Comparison of cellulose products obtained by Jenkins's method with those given by the Cross and Bevan procedure without pre-treatment.*

Straw	Jenkins cellulose in 100 g. straw	Furfurald. in 100 g. Jenkins cellulose	Apparent lignin in 100 g. Jenkins cellulose	C. and B. cellulose in 100 g. straw	Furfurald. in 100 g. C. and B. cellulose	Apparent lignin in 100 g. C. and B. cellulose
Oats A	41.9	9.1	4.8	53.5	16.8	3.0
Wheat A	47.5	9.9	7.8	55.2	17.7	3.1
Wheat B	45.1	10.4	4.6	56.7	18.3	2.9
Barley	35.2	8.9	3.9	47.7	—	3.3
Rye	51.9	10.9	6.6	60.2	19.0	2.4

Apart from the serious losses of cellulosan, to which attention has been directed above, it will be seen that the lignin figures given by celluloses prepared by the Jenkins procedure are hardly satisfactory. Even those obtained on the Cross and Bevan products are not as low as expected, an observation which will be dealt with in more detail below.

Therefore, although the method proposed by Jenkins [1930] may be suitable as a rapid and arbitrary procedure for determining a certain fraction of straws, a fraction not dissimilar from that given by the old-established crude fibre determinations, it cannot be regarded as providing a real indication of the cellulosic fabric of the material. It is faulty in two respects; firstly that the cellulosan fraction is very heavily attacked, and secondly that the product is not completely delignified.

The rôle of sulphite in the determination of cellulose by a chlorination procedure.

The chlorination methods of cellulose determination, as previously stated, depend on alternate exposures to gaseous chlorine and extraction with boiling sodium sulphite in which the lignin-chlorine complex is soluble. At the same

time the hemicelluloses are removed by the boiling sulphite. It seemed to be of interest therefore to determine to what extent the chlorination itself is important. Samples of oat straw were given 1, 3 and 6 treatments with boiling sulphite for 20 minutes and the residues analysed for lignin and total furfuraldehyde. At the same time three other samples were given 1, 3 and 6 gaseous chlorinations for 10 minutes, each followed by boiling with sulphite for 20 minutes. The results are given in Table IV. Six neutral sulphite

Table IV. *Effect on oat straw of sulphite boils with and without chlorination.*

Treatment	Residue	Furfurald. on residue	Apparent lignin on residue	Furfurald. on original straw basis	Apparent lignin on original straw basis
1 chlorination*	73.4	19.2	12.2	14.1	8.9
1 sulphite boiling				(11.2)	(55.2)
1 sulphite boiling	75.6	20.4	14.8	15.4	11.2
				(2.8)	(43.8)
3 chlorinations	58.2	16.4	5.7	9.5	3.3
3 sulphite boilings				(39.9)	(83.4)
3 sulphite boilings	68.4	19.6	12.5	13.4	8.5
				(15.5)	(57.2)
6 chlorinations	52.2	17.2	4.4	9.0	2.3
6 sulphite boilings†				(43.5)	(88.5)
6 sulphite boilings	62.7	19.7	8.5	12.4	5.2
				(22.2)	(74.0)

* Chlorination by gaseous Cl₂ 10 mins., 3 % sulphite boiling—20 mins.

† Still gave sulphite reaction for lignin.

Figures in brackets give percentage loss by treatment.

treatments alone remove 74 % of the lignin but only 22 % of the total furfuraldehyde-yielding constituents. The effect of the chlorination is greatly to expedite the removal of lignin, since at the end of the third chlorination as much as 83 % has been extracted. The chlorine however also has a marked effect on the removal of hemicelluloses, as indicated by the furfuraldehyde figures. Three chlorinations lower the furfuraldehyde yield by 40 % while three sulphite treatments alone only remove 15 %. This effect of chlorine in assisting in the removal of the hemicelluloses was unexpected and has not been commented upon before. It seems that the chlorination is at least as important in this respect as in removing lignin. Its mode of action is not understood at present, but will be the subject of further investigation. Neutral sulphite without chlorination has been proposed as a method of delignification of pulps but has not been found satisfactory, since high pressures and concentrations are necessary to obtain a satisfactory product [Rawling and Staidl, 1925].

Proposed new method for the determination of cellulose.

In view of the many chlorinations to which it is necessary to subject a cereal straw in order to obtain satisfactory delignification without pre-treatment, an attempt was made to devise a method more suited to this type of material. Jenkins [1930] observed that lignin could be removed in part by the use of sodium hypochlorite. It was found, however, that the delignifying effect could be enhanced by following the hypochlorite treatment by boiling with sodium sulphite, just as is done in the Cross and Bevan procedure. No colour reaction with sulphite, such as is given after treatment with chlorine gas, is observed.

The mode of action of the hypochlorite is not clear, and it can only be supposed that a soluble additive compound with the whole lignin molecule is obtained. After preliminary boiling with water, about 2 g. straw were allowed to stand for 15 minutes in 100 cc. water to which had been added 5 cc. hypochlorite containing approximately 15 % available chlorine¹. The straw was then filtered off on cloth and boiled for 20 minutes with 100 cc. 3 % sodium sulphite. This process was carried out one, two, three, four and six times on a sample of oat straw, with the results given below. Though the products after 4 and 6 hypo-

Table V. *Effect on oat straw of neutral hypochlorite treatments followed by sulphite boiling.*

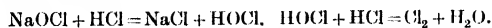
Procedure	Cellulosic product	Apparent lignin % on cellulose
1 hypochlorite treatment	69.92	14.8
2 hypochlorite treatments	63.24	9.2
3 " "	54.80	9.5
4 " "	54.04	6.9
6 " "	53.66	4.7
C. and B. 9 chlorinations without pre-treatment	53.99	3.1

chlorite treatments were excellent in appearance and retained the original structure they did not compare favourably in lignin content with those obtained by the Cross and Bevan procedure. Moreover, when exposed to gaseous chlorine, the product after 6 neutral hypochlorite treatments gave an intense purple reaction for lignin on addition of sulphite. Further, on subjecting different straws to 4 hypochlorite treatments, the effectiveness of the treatment was found to vary considerably, as will be seen in Table VI.

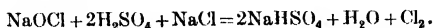
Table VI. *Comparison of products of four neutral hypochlorite treatments with those obtained by the Cross and Bevan method.*

Straw	Hypochlorite method		Cross and Bevan method	
	Cellulose product	Lignin on product	Cellulose product	Lignin on product
Oats A	54.0	6.9	53.4	3.1
Oats B	53.1	4.3	53.5	3.0
Wheat A	56.2	5.2	55.2	3.1
Wheat B	59.6	7.4	56.7	2.9
Barley	48.3	6.7	47.7	3.3
Rye	65.6	9.1	60.2	2.4

¹ An excellent and inexpensive solution of sodium hypochlorite may be obtained from Messrs Laporte of Luton. It contains hypochlorite equivalent to 15-17 % available chlorine, together with about 14 % NaCl, 1 % NaOH and 1 % Na₂CO₃. The strength of the hypochlorite may readily be determined by titration with thiosulphate in the presence of KI and H₂SO₄. The available chlorine or chlorine liberated on acidification represents twice the amount actually present as NaOCl, as shown by the equations



Owing to the presence of NaCl in the solution the same relationship holds on acidification with sulphuric or any other strong acid.



The equations given above have been verified experimentally.

In the absence of any easy and reliable test for lignin, clearly a method which involved a different number of treatments with different materials could not be very satisfactory. Accordingly it was decided to introduce a treatment with free chlorine so that the sulphite colour reaction for lignin might be employed and in the hope that the lignin might more completely be removed. The free chlorine was obtained by adding to hypochlorite an amount of sulphuric acid somewhat in excess of the equivalent so that hypochlorous acid should not be present to any extent. When cold sulphite is added to the straw from this acid hypochlorite treatment, if lignin is still present, a red purple colour develops just as if exposed to chlorine gas. In straws which have previously undergone a treatment with neutral hypochlorite and sulphite, the colour is more definitely purple than the red-brown colour usually obtained after chlorine gas. This suggests that the neutral hypochlorite removes some material or some portion of the lignin masking the purplish tint. Many experiments were carried out to determine the most satisfactory number combination of neutral¹ and acid chlorination treatments. To be practical it was considered that the number ought not in normal circumstances to exceed five, and accordingly the estimations were stopped at that point whether or not a sulphite reaction for lignin was still given. The results of some of the combinations of neutral and acid hypochlorite treatments are shown in Table VII.

Table VII. *Comparison of results of various combinations of neutral and acid hypochlorite treatment of oat straw.*

Treatment*	Cellulose on straw	Apparent lignin on cellulose	Lignin reaction at end
C. and B. 9 chlorinations	53.4	3.1	None
A.A.A.A.A. (2 mins. A.)	55.6	3.6	None
N.N.A.A.A. (2 mins. A.)	54.1	3.7	None
A.N.A.N.A. (2 mins. A.)	55.5	3.9	Slight
N.N.N.A.A. (2 mins. A.)	56.1	3.5	Definite
N.N.A.N.A. (2 mins. A.)	54.7	3.8	Trace
A.N.N.A.A. (2 mins. A.)	56.0	3.7	Strong
A.A.A.A.A. (15 mins. A.)	54.8	3.4	Slight
N.N.A.A.A. (15 mins. A.)	53.6	3.1	None
N.N.A.A.A. (5 mins. A.)	55.0	3.3	Slight
N.N.A.A.A.G. (5 mins. A.)	53.1	3.0	None
N.N.A.A.A. (10 mins. A.)	53.5	3.5	None

* N. 5 cc. NaOCl (15 % avail. Cl.) to 100 cc. water for 5 minutes, filtered and followed by sulphite boiling for 10 minutes.

A. 5 cc. NaOCl (3 % avail. Cl.) to 100 cc. water + 2 cc. 20 % H₂SO₄, filtered and followed by sulphite boiling for 10 minutes.

(†. Exposure to chlorine gas for 5 minutes.

N. As N. but for 10 minutes.

Chlorination with acid hypochlorite under similar conditions was found to be harmless to pure cellulose. Finely ground filter-paper given three such treatments for 5 minutes followed each time by boiling for 10 minutes with sulphite suffered a loss of only 2.7 %. Controls which were given three sulphite boilings without chlorinations lost precisely the same amount.

Arising from Table VII, the following method was adopted and found to be applicable not only to straws but also to woods. The material should be in a

¹ The treatments are spoken of as neutral and acid respectively for the sake of clarity. The neutral treatments are in fact faintly alkaline by reason of the free alkali in the sodium hypochlorite, necessary for stability.

uniform and fine state of division; in the case of woods 60–80 mesh appears to be most suitable, while in the case of straws 40–60 seems preferable. Woods must be given an alcohol-benzene extraction but this is unnecessary with cereal straws. About 2 g. material are brought to the boil in 100 cc. 3 % sulphite and filtered off on fine smooth poplin stretched over a Büchner funnel about 6 cm. in diameter and closely fastened either in a ring or with tight rubber bands. This preliminary sulphite boiling is advantageous not only in ensuring thorough wetting of the material but also in that a certain amount of lignin is removed, as shown in Table IV. Furthermore straws boiled with sulphite often filter much more readily than if treated only with water. The filtered material is then removed to a beaker by means of a spatula and jet from a wash-bottle. After making up the filtered straw to 100 cc., 5 cc. sodium hypochlorite (approx. 15 % available chlorine) are added and allowed to stand for 10 minutes. The material is filtered off, again transferred to a beaker and made up to 50 cc. with water; 50 cc. 6 % sodium sulphite are added, and the whole boiled for 20 minutes. It is usually convenient to bring to the boil over a flame and then to stand the beaker in a boiling water-bath, as bumping is frequently serious in the later stages. The treatment with hypochlorite is repeated a second time and followed by a sulphite boiling as before. The material, which by this time is much lighter in colour, is again suspended in 100 cc. water, and 5 cc. dilute hypochlorite (3 % available chlorine) are added, together with 2 cc. 20 % sulphuric acid. Chlorine is evolved and the material frequently turns yellow, just as it does in the Cross and Bevan process with gaseous chlorine. After standing for 10 minutes out of direct sunlight it is filtered, made up to 50 cc. and sulphite added as before. An intense purple coloration is given indicating the presence of lignin. This colour disappears on heating and the solution then becomes brown. The treatments with acid hypochlorite are continued as long as a positive reaction for lignin is given on addition of sulphite. In the case of some straws two acid treatments are sufficient. The process should not be continued longer than is necessary as indicated by this reaction. Sulphite is removed by suspension in 200 cc. or so of hot water, and after thorough washing the cellulose product is transferred to a Gooch crucible containing a small circle of cloth. It is dried to constant weight at 100°.

Chlorination under these conditions is without serious effect on pure cellulose. Samples of Whatman No. 1 filter-paper, finely ground, were given in duplicate the treatment described above but prolonged to the extent of 5, 7 and 10 acid chlorinations to determine the effect, if any, of gross over-chlorination. The recoveries from 1 g. were practically identical and equal to the amount obtained after 6 alternate cold and boiling-water extractions. The latter treatment represents water-soluble material *plus* mechanical losses in handling. These results are given in Table VIII.

Table VIII. *Effect of over-chlorination on pure filter-paper.*

Treatment	Amount recovered from 1 g. cellulose
2 N. 3 A.*	0.958
2 N. 5 A.	0.951
2 N. 7 A.	0.956
2 N. 10 A.	0.952
6 BW. 5 CW.†	0.952
Boiled in water 2 hours	0.991

* Times and concentrations as given in Table VII.

† Boiled for 20 minutes with water, filtered and allowed to stand in cold water for 10 minutes.

Table IX shows results obtained for certain straws and woods by this method, compared with the figures obtained by the Cross and Bevan procedure without pre-treatment. The cellulose figures are the averages of several determinations, while lignin and furfuraldehyde estimations were carried out only in duplicate. From this evidence it is claimed that the method gives results strictly comparable with those of the Cross and Bevan determination without pre-treatment, and that the product represents as closely as is possible the natural cellulosic tissue of the plant freed from encrusting substances. It possesses certain general advantages in that it dispenses with the use of chlorine gas and needs no permanent apparatus. With a sufficiently large water-bath to take the sulphite extractions after boiling, one worker can carry on as many as 16 determinations and, unless the material is especially resistant, carry them all through to completion in the course of a day. For cereal straws the method is preferable, since the number of chlorinations necessary is lessened, and the product does not become gelatinous or difficult to handle.

Table IX. *Comparison of results obtained on straws and woods by the Cross and Bevan method without pre-treatment and by the new method.*

Material	Cross and Bevan method				New method			
	No. of chlorina- tions	Cellulose %	Furfurald. on cellulose %	Apparent lignin on cellulose %	No. of chlorina- tions*	Cellulose %	Furfurald. on cellulose %	Apparent lignin on cellulose %
Straws and woods								
Oats A	7	53.4	17.4	3.1	2 N. 3 A.	53.5	17.2	3.5
Oats B	7	53.5	16.8	3.0	2 N. 3 A.	52.5	17.0	2.6
Wheat A	7	55.2	17.7	3.1	2 N. 3 A.	55.5	17.6	1.6
Wheat B	6	56.7	18.3	2.9	2 N. 3 A.	56.3	18.2	2.4
Rye	7	60.2	19.0	2.4	2 N. 4 A.	59.8	18.7	2.4
Oak (Danzig)†	10	53.2	18.1	0.3	2 N. 5 A.	53.9	18.2	0.7
Beech†	8	58.8	15.5	1.0	2 N. 5 A.	59.4	15.5	2.1
Scotch Pinet	8	60.2	5.2	0.8	2 N. 5 A.	60.8	5.5	0.2
Sitka Spruce†	6	60.8	3.3	0.2	2 N. 5 A.	65.9	3.2	0.1

* N. Neutral hypochlorite treatment. A. Acid hypochlorite treatment.

† Results expressed on material extracted with alcohol-benzene.

The method is easily applicable to large scale preparations of natural celluloses, and very satisfactory products have been obtained from a number of materials, both woods and straws. To obtain any quantity of cellulose by the Cross and Bevan procedure is a slow and laborious business in that the amount which can be chlorinated at any one time is limited. By the new method 200-300 g. can be treated at a time.

The determination of lignin in cellulose products.

One of the vital requirements in a method of determination of cellulose is, of course, that lignin be completely removed. Hawley and Wise [1926] state that small amounts of lignin are often tenaciously retained by the Cross and Bevan cellulose product, yet it is surprising that cellulose methods have rarely been subjected to this ultimate test, namely the lignin content of the product. Ritter and Fleck [1924] compare figures for lignin in celluloses treated with chlorine for long and short periods. They show that with the longer treatments the lignin remaining is only slightly lessened, but they fail to comment on the significance of the lignin figures themselves. Chlorinations were continued till no colour was given on addition of sulphite, but even so the lignin content varied from 1.0 to 1.5 %. In this work a lignin determination was carried out on every cellulose product, since the appearance is not a satisfactory criterion.

Preparations of excellent colour may nevertheless contain appreciable quantities of lignin. This is very true of products obtained by the Jenkins method in which delignification is incomplete. In Table IX is given the lignin content of products obtained from straw by both the Cross and Bevan and the new procedure, continued until no colour is given with sulphite. The method adopted was to add to the cellulosic material 20 cc. 72 % sulphuric acid, and after repeated stirring to allow the whole to stand overnight in a cool place, the temperature not being allowed to exceed 20°. After dilution with 250 cc. distilled water, the solution was boiled for half an hour, allowed to settle and filtered. The longer period with 72 % acid and short hydrolysis at an acid concentration of about 9.5 % is preferred to the present method advocated by the U.S. Forest Products Laboratory, which involves only a brief treatment with strong acid and 4 hours' hydrolysis after dilution to an acid concentration of 3 %. By the method adopted the Cross and Bevan celluloses from straws contained approximately 3 % lignin, and this figure could not be lowered appreciably by over-chlorination. Celluloses prepared by the new method were uniformly a little lower, but, even so, averaged about 2.5 %. Inasmuch as in both cases the wood celluloses were very considerably lower in lignin content it seemed that this high lignin figure might be apparent rather than real, and the reason for it inherent in the nature of the straw.

Recently a number of investigators [*e.g.* Wise and Fairbrother, 1931; Sherrard and Harris, 1932] have pointed out some of the defects of the 72 % sulphuric acid method for lignin, and some have made diametrically opposed recommendations as to the conditions under which the determination should be carried out. Ritter, Seborg and Mitchell [1932] suggest a brief treatment at 20°, while Peterson, Walde and Hixon [1932] prefer lower temperatures for a longer period and recommend that the reaction should be carried out in an ice-box. All are agreed that acid of this strength causes a certain amount of charring or caramelisation of the carbohydrate material, and that this may produce serious errors if the temperature rises above about 20°. Hawley and Wiertelak [1931] and Hawley and Harris [1932] have shown that a lignin-like material may be produced by exposure of cellulose or wood to a temperature of 135° for several days. Of the carbohydrate material concerned, the cellulosan, which is pentose in nature in the hardwoods and largely hexose in softwoods, suffers the greatest loss. Since, by a violent process of dehydration by heat, apparent lignin can be obtained in considerable amount from the cellulosans, it seemed possible by analogy that smaller quantities of some similar material might be formed by the action of 72 % sulphuric acid as used in the lignin determination. In some work, as yet unpublished, Norman has found that the cellulosans are readily removable by dilute acid hydrolysis. Lignin presumably is unaffected by dilute acid, since prolonged boiling in acid concentration of 4 % or more is generally accepted as a part of the estimation procedure. If, then, this theory is correct, the apparent lignin content of a cellulose from which the cellulosan has been removed by hydrolysis would be reduced as a result of the treatment. A sample of oat-straw cellulose prepared by the proposed new method had an apparent ash-free lignin content of 3.47 %. On boiling for 1 hour with 3 % H_2SO_4 , a treatment known to remove a part, but not all, of the cellulosan, the cellulose suffered a loss of 17.1 % and the product had then an apparent lignin content (on the basis of the original cellulose present) of 2.37 %. Similarly a sample of oat-straw cellulose, prepared by the Cross and Bevan process and containing 3.0 % apparent lignin before hydrolysis, gave under the same treatment a product containing only 2.23 %. The effect of the

removal of part of the cellulosan in both cases therefore was to lower the apparent lignin content by nearly a third. It seems clear that from the pentose material some product is being produced insoluble in boiling dilute acid and therefore determined as lignin. The 72 % sulphuric acid solution of the cellulose after removal of cellulosan was, incidentally, very markedly lighter in colour than that of the untreated material. The effect of hydrolyses with different strengths of acid for various times in lowering the apparent lignin content was next studied. Cellulose was prepared from wheat straw by the hypochlorite method, two neutral and three acid treatments being given. The product had an apparent lignin content of 2.4 %. 1.5 g. samples were hydrolysed under several conditions, filtered, dried and weighed and the lignin content of the residues then determined. The results are given in Table X. Treatment with

Table X. *Effect of hydrolysis of cellulosans on the apparent lignin content of a wheat-straw cellulose containing 2.4 % lignin.*

H ₂ SO ₄ strength %	Time in hours	Loss %	Furfural, in solution %	Apparent lignin %
1.0	$\frac{1}{2}$	15.5	5.52	2.2
3.0	1	23.6	—	1.9
3.0	2	24.8	—	2.1
3.0	3	25.8	10.44	1.8
5.0	1	25.2	—	1.7
5.0	2	27.2	10.52	1.9
10.0	3	35.3	12.36	1.6

10 % acid, which, of course, results in appreciable hydrolysis of the cellulose itself as well as complete removal of the cellulosans, lowered the apparent lignin figure by a third to 1.6 %. Since this is still an amount higher than that found for most woods, it seemed desirable to investigate the nature of this residual apparent lignin, and to attempt to decide whether it is true lignin or secondary material produced in the course of the reaction. If true lignin, then it ought, perhaps, to be removable by gross over-chlorination and repeated extractions, whereas, if secondary material, such violent treatments should leave the amount very little changed.

Wheat straw of apparent lignin content 20.3 % was treated in a series of 1.5 g. samples by the new method for cellulose determination. All the samples were given 2 neutral hypochlorite treatments, and then in pairs 1, 2, 3, 5 and 7 acid hypochlorite treatments respectively, each being followed by sulphite boiling. The apparent lignin was determined directly in one of the two duplicates and in the other after hydrolysis for 1 hour with 5 % H₂SO₄ to remove the cellulosan, as described above. The results are presented in Table XI. It will be seen that the apparent lignin content is appreciably lowered in all cases but the last by removal of the cellulosan. Even on gross over-chlorination to the extent of five extra acid hypochlorite treatments after the last trace of reaction with sulphite, the apparent lignin is quite appreciable, amounting to about 1.5 % on the original straw basis. Since it might have been supposed that these continued chlorinations would remove any residual lignin, this residue might be ascribed to material secondarily produced from the cellulose itself by charring or caramelisation. This view, however, is not supported by evidence from other materials. Results obtained with wood celluloses are of a considerably lower order, and both hardwood and softwood cellulose products have been obtained, as shown in Table IX, with an apparent lignin content as low as 0.1 to 0.5 % on the basis of the cellulose. Other hexosan material, such as

Table XI. *Removal of apparent lignin from wheat straw at successive stages of chlorination.*

All results expressed on original dry straw. (Apparent lignin = 20.3 %.)

Treatment	Colour reaction with sulphite	Product on chlorination %	Residue after hydrolysis with 5 % H_2SO_4 %	Apparent lignin in chlorination product	Apparent lignin in hydrolysis residue
—	—	—	57.23	—	15.2
2 N.	—	68.56	48.04	8.3	6.7
2 N. 1 A.	Strong	54.91	42.81	3.3	2.1
2 N. 2 A.	Trace	53.03	41.12	3.1	1.7
2 N. 3 A.	Nil	51.45	41.02	3.2	2.0
2 N. 5 A.	Nil	48.73	41.02	3.0	1.9
2 N. 7 A.	Nil	48.03	40.84	1.5	1.7

pure filter-paper or starch similarly yield only a trace of apparent lignin. It is not natural to suppose that straw cellulose is more susceptible to charring or caramelisation than other celluloses, since the magnitude of the apparent lignin figures is so different. The residue obtained, therefore, must be either lignin so tenaciously retained as to be unaffected by chlorination and sulphite extraction or some other acid-resistant constituent of unknown composition. This point will be tested further and an attempt made to characterise or extract this material by some such means as a modification of the glycol method of Hibbert and Rowley [1930].

Additional support for the view that from pentose material there is produced some material with the acid-resistant properties of lignin was obtained by carrying out that determination on filter-paper alone, filter-paper *plus* glucose and filter-paper *plus* xylose. 1 g. of filter-paper alone yielded 0.02 mg. apparent lignin, with the addition of an equal weight of glucose 0.03 mg. apparent lignin, but with the addition of only 0.25 g. xylose as much as 5.5 mg. apparent lignin.

Although somewhat outside the scope of this work the information obtained on the formation of apparent lignin from pentosan groupings may be of great importance in the ordinary determination of lignin in untreated materials. W. G. Campbell in a private communication has expressed the opinion that the hemicelluloses are a source of error in the lignin determination. It may therefore be necessary to modify the method by the introduction of a mild hydrolytic treatment for the removal of these constituents. In Table II is shown the effect of a brief extraction of oat straw with HCl, the apparent lignin content

Table XII. *Effect on apparent lignin content* of preliminary hydrolysis of straws and woods (5 % H_2SO_4 for 1 hour).*

Results expressed on 100 g. material.

Straw	Apparent lignin %	Loss on hydrolysis %	Apparent lignin after hydrolysis
Oats	18.4	42.5	12.6
Wheat	19.3	37.5	13.7
Barley	15.7	49.4	11.2
Beech†	22.8	26.7	19.2
Scotch pine†	24.1	23.6	23.7

* Determined by treatment for 16 hours with 72 % H_2SO_4 , dilution to 3 % and boiling for 2 hours. These figures therefore are directly comparable with those obtained by other workers.

† Results expressed on material extracted with alcohol-benzene.

falling from 19.9 to 13.5 %, a loss of no less than a third as a result of a treatment which would presumably not affect true lignin. Similarly some of the straws and woods employed were boiled for 1 hour with 5 % sulphuric acid and almost equally large losses in apparent lignin content noted, as shown in Table XII.

These great differences make it clear that the whole question of the determination of lignin by the 72 % sulphuric acid method must be critically re-examined, since, if the indications given here are correct, the quoted lignin figures for straws and hardwoods are too high as a result of secondarily produced material from the pentose groupings. Those for the softwoods, in view of the smaller content of pentose material, are probably more nearly accurate. This may be seen from the figures given for beech and Scotch pine in Table XII.

SUMMARY.

1. The cellulossans or polysaccharide material very intimately associated with "pure" cellulose in nearly all natural celluloses should remain intact in any satisfactory method of cellulose estimation.

2. Alkaline pre-treatment, as often recommended, is objectionable in that a portion of the cellulossan is removed, even though delignification is much assisted.

3. Jenkins's method of cellulose determination is unsatisfactory for two reasons. First, the preliminary treatments with alkali and acid alternately result in a very severe loss of cellulossan, and second, the product is incompletely delignified by the subsequent treatments with neutral hypochlorite.

4. In the chlorination methods of cellulose determination the chlorine is at least as important in causing the solution of hemicelluloses during the subsequent treatment with boiling sulphite as in assisting in the removal of lignin. Sulphite alone results in extensive, but not complete, delignification without heavy loss of encrusting polyuronide hemicelluloses.

5. Neutral hypochlorite treatments followed by sulphite boilings will effect a fairly complete delignification of plant materials.

6. A new method is proposed for both straws and woods involving first two treatments with neutral hypochlorite and then three or more with acid hypochlorite, each followed by boiling with sodium sulphite. The results obtained are almost identical with those obtained by the Cross and Bevan method without pre-treatment. The proposed method is more rapid with straws in that it involves fewer treatments. It is readily applicable to large scale preparations.

7. The products from cereal straws given by both the Cross and Bevan and the new methods are not entirely free from lignin. The lignin figure obtained by the use of 72 % H_2SO_4 on such products is, however, not accurate, since appreciable quantities of apparent lignin are produced from the cellulossan when pentose is nature. Upon removal of this fraction by acid hydrolysis the lignin figures are reduced by a quarter to a third, the residue being unaffected by over-chlorination. It must therefore be either true lignin very tenaciously held, or else some other resistant material of unknown composition.

8. In view of the production of apparent lignin from pentosan groupings by 72 % H_2SO_4 , the figures obtained for lignin on many natural materials may be unreliable. A brief preliminary acid hydrolysis of straws results in the lowering of the apparent lignin content by 25-30 %. Hardwoods similarly are likely to give figures which are too high.

We are indebted to Mr L. W. Samuel of this Department for assistance with some of the analytical work involved and to Mr W. G. Campbell of the Forest Products Research Laboratory, Princes Risborough, Bucks, for his helpful criticisms of the manuscript.

REFERENCES.

- Cross and Bevan (1895). *Cellulose*. (London.)
Dorée (1933). *The methods of cellulose chemistry*, p. 333. (London.)
Hawley and Harris (1932). *Ind. Eng. Chem.* **24**, 873.
—— and Norman (1932). *Ind. Eng. Chem.* **24**, 1190.
—— and Wiertelak (1931). *Ind. Eng. Chem.* **23**, 184.
—— and Wise (1926). *The chemistry of wood*. (New York.)
Hibbert and Rowley (1930). *Canadian J. Res.* **2**, 357.
Jenkins (1930). *Biochem. J.* **24**, 1428.
Norman (1929). *Biochem. J.* **23**, 1353.
—— (1933). *J. Agric. Sci.* **23**, 216.
Peterson, Walde and Hixon (1932). *Ind. Eng. Chem. Anal. Edit.* **4**, 216.
Rawling and Staidl (1925). *Paper Trade J.* **81**, 49.
Renker (1910). *Über Bestimmungsmethoden der Cellulose*. (Berlin.)
Ritter and Fleck (1924). *Ind. Eng. Chem.* **16**, 147.
—— Seborg and Mitchell (1932). *Ind. Eng. Chem. Anal. Edit.* **4**, 202.
Sherrard and Harris (1932). *Ind. Eng. Chem.* **24**, 103.
Strong (1928). *J. Soc. Chem. Ind.* **47**, 87 T.
Wise and Fairbrother (1931). *Ind. Eng. Chem. Anal. Edit.* **3**, 253.

CCLXXXIII. THE DETERMINATION OF LIGNIN.

I. ERRORS INTRODUCED BY THE PRESENCE OF CERTAIN CARBOHYDRATES.

By ARTHUR GEOFFREY NORMAN
AND SAMUEL HARRY JENKINS.

*From the Biochemistry Section and Fermentation Department,
Rothamsted Experimental Station.*

(Received October 29th, 1934.)

THE determination of lignin, though apparently a simple matter, is one of the least satisfactory of the analyses commonly carried out on plant materials and woods. Practically all the methods generally used involve the solution and hydrolysis of all other plant constituents and the assumption that the residue after such treatment is exclusively lignin. Various workers from time to time have pointed out that this is not the case, but in spite of this vital objection, considerable reliance has been placed on figures so obtained. There has similarly been some conflict as to the precise procedure to be adopted, inasmuch as different values are given by different methods. The reasons for these differences have not usually been given and are little understood.

In this work an attempt has been made to study some of the factors which disturb the lignin determination, in order that the best possible method may be selected for any particular material. It is not necessarily to be expected that one procedure will be found universally suitable for all classes of material.

The generally accepted methods employ a strong mineral acid to dissolve cellulose and other constituents, either 72 % sulphuric acid in some modification or variation of the Ost and Wilkening [1910] method, or fuming hydrochloric acid (42 %), following the procedure of Willstätter and Zeehmeister [1913]. Other things being equal, the 72 % H_2SO_4 is less unpleasant to handle and is consequently most frequently employed. The sulphuric acid method has been used exclusively in these investigations, and no attempt has been made to compare the results obtained with those given by the concentrated HCl method as developed by Phillips [1932]. Probably the best-known modification of the Ost and Wilkening procedure is that employed for many years by the U.S. Forest Products Laboratory in which the material, after alcohol-benzene extraction, is treated at room temperature for 17 hours with 72 % sulphuric acid, the mixture then being diluted till the acid concentration is 3 % and boiled for 2 hours. The lignin is filtered off and weighed. Sherrard and Harris [1932] more recently showed that the amount and properties of the apparent lignin obtained by this procedure are altered by variations in temperature during the 72 % acid treatment, and accordingly the method was considerably modified by Ritter *et al.* [1932]. Following the initial alcohol-benzene extraction they introduced a treatment with boiling water for 3 hours. This is stated to cause the lignin residue to be "lighter in colour, the yield lower, and filtration and washing facilitated." No evidence is given as to the nature of the material

removed, though it is presumed to be either insoluble in sulphuric acid or converted into insoluble products by the acid treatment. The subsequent 72 % acid treatment is continued for only 2 hours and the temperature maintained at 20°, it being shown that higher temperatures cause caramelisation. After dilution to 3 % acid, the solution is boiled for 4 hours and filtered as before. Peterson *et al.* [1932] similarly showed that the temperature should not be allowed to be high during the 72 % acid treatment, and in their case recommended an 18-hour treatment at 4°, though there was little difference between the results obtained at 4 and 15°. Cohen and Dadswell [1931] were unable to obtain satisfactory results for certain Australian woods, particularly Eucalypts, by existing methods and proposed a pretreatment of the wood with *N*/8 sodium hydroxide at 100° for 80 minutes to remove extraneous matter causing high results. It has not been conclusively shown that such a treatment does not at the same time remove some lignin.

Recently Phillips [1934] has compared on a number of different materials the original U.S. Forest Products Laboratory method, that of Peterson *et al.* [1932], an older method of Schwalbe [1925] employing a mixture of 72 % H_2SO_4 and concentrated HCl, and his own method [1932] which makes use of 42 % HCl. Unfortunately the later procedure of Ritter *et al.* [1932] was not included. Further, all the materials received a pretreatment by extraction with hot water, a process not a part of any method but that of Phillips [1932]. He ranked the methods according to yield, the lowest being that of Phillips [1932], and then in order, Schwalbe, Peterson *et al.* and the U.S. Forest Products Laboratory method, the last giving very considerably higher figures in most cases.

Some of these methods and modifications will be discussed later in the light of the experimental work here presented. In several cases changes in procedure recommended by various workers seem to have been made without any clear appreciation of the reasons underlying their effects.

EXPERIMENTAL.

(a) *Effect of sugars on apparent lignin yields.*

In a paper on the determination of cellulose, Norman and Jenkins [1933] remarked that cellulose preparations from straws giving no test for lignin often yielded about 3 % apparent lignin and showed that this figure could be reduced by treating the cellulose in such a way that the cellulosan fraction was to a great extent removed. They presumed that some form of condensation occurred in the presence of the 72 % acid with the production of an insoluble and unhydrolysable product weighed as lignin, and gave evidence to show that xylose units were responsible for this phenomenon. This has been confirmed. Table I shows the effect of the addition of xylose in various amounts on the apparent lignin content of hydrolysed straw, so treated as to remove practically all hemi-cellulose material.

All results in this, and later Tables, are the average of two or more closely agreeing determinations, and all lignin determinations are corrected for ash. The straw and other materials used were finely ground and passed through a 60-mesh sieve.

It appears that the disturbance produced by the addition of the sugar is very appreciable but not proportional to the increments added, or necessarily identical in different experiments,

Table I. *Addition of xylose to hydrolysed oat straw (5 % acid for 1 hour).*

16 hours with 72 % acid, temp. <20°; diluted to 3 % and boiled for 2 hours.

	Apparent lignin content % of straw	Increase per 0.1 g. xylose added
Straw A (0.5 g.) alone	22.11	—
„ „ +0.1 g. xylose	24.98	2.87
„ „ +0.25 g. „	29.00	2.76
„ „ +0.5 g. „	34.50	2.48
Straw B (0.5 g.) alone	28.00	—
„ „ +0.25 g. xylose	31.16	1.26
„ „ +5 g. „	34.10	1.22
„ „ +1.0 g. „	36.94	0.89
„ „ +2.0 g. „	40.54	0.63

The following other sugars were tested, alone and in the presence of cellulosic material: arabinose, glucose, mannose, galactose, fructose and sucrose. Of these only arabinose, fructose and sucrose gave rise to any apparent lignin, and the amount from arabinose was very small. From filter-paper *plus* 1 g. sucrose, treated with 72 % H₂SO₄ for 16 hours, 22 mg. apparent lignin were obtained after dilution and boiling; from 1 g. fructose, similarly treated, 48 mg. and from 1 g. arabinose, 8 mg. Table II gives some observations on the addition of sucrose and arabinose to straw, the sucrose presumably causing a disturbance in the apparent lignin content by reason of its fructose unit.

Table II. *Addition of sucrose and arabinose to straws.*

16 hours with 72 % acid, temp. <20°; diluted to 3 % and boiled for 2 hours.

	Apparent lignin content % of straw	Increase per 0.1 g. sugar added
0.5 g. straw (hydrolysed bean straw)	22.1	—
„ „ +0.5 g. sucrose	25.7	0.72
„ „ +1.0 g. „	28.0	0.59
0.5 g. straw (oat straw)	18.5	—
„ „ +0.5 g. arabinose	18.9	—
„ „ +1.0 g. „	18.9	—

The disturbance produced by sucrose, though considerable, is not as great as that caused by xylose and likewise is not proportional to the amount present. That given by arabinose is very small. An explanation of this difference will be given later.

Very recently Hilpert and Littman [1934] have recorded the same observations, that these sugars with strong acid yield insoluble products, but in their experiments the time of contact was 48 hours, and the residues were consequently higher.

Since free xylose, or its fission products, in the presence of concentrated sulphuric acid condenses to form an insoluble material, it was reasonable to

Table III. *Addition of xylan to oat straw.*

16 hours with 72 % acid, temp. <20°; diluted to 3 % and boiled for 2 hours.

	Apparent lignin content of straw %
0.7 g. straw alone	19.71
„ „ +0.3 g. xylan	22.07

suppose that the polysaccharide xylan would also do so. This was proved by testing a crude xylan preparation obtained by the cold alkaline extraction of a prepared straw cellulose (Table III).

Similarly, increases in apparent lignin were obtained when a crude hemicellulose sample containing about 50 % pentose, prepared from oat straw, was employed. Like practically all hemicellulose preparations this was not absolutely free from lignin, which was determined by boiling with 3 % acid for 2 hours. The insoluble residue, after correction for ash, was regarded as lignin and found to be 4.27 %.

Table IV. *Addition of crude hemicellulose to hydrolysed oat straw (5 % acid, 1 hour).*

16 hours with 72 % acid, temp. <20°; diluted to 3 % and boiled for 2 hours.

	Apparent lignin g.	Lignin in hemi- cellulose g.	Apparent lignin from straw g.	Apparent lignin on straw %
0.5 g. straw alone	0.1317	—	0.1317	26.34
" " + 0.1 g. hemicellulose	0.1450	0.0043	0.1407	28.14
" " + 0.3 g. "	0.1525	0.0128	0.1397	27.94
" " + 0.5 g. "	0.1691	0.0213	0.1478	29.56
0.3 g. hemicellulose alone	0.0203	0.0128	—	—

Unfortunately it was not possible to test the effect of the uronic acids upon lignin yields, and until this is done they must, from their very nature, be regarded as likely causes of disturbance. Additions of pectin, which contains nearly 80 % of tetragalacturonic acid anhydride, did not produce any increase in apparent lignin.

(b) *Effect of duration of acid treatment on apparent lignin yields.*

In all the experiments quoted above, the 72 % acid was allowed to act for 16 hours at a temperature less than 20°. The work of Sherrard and Harris [1932] on the effect of temperature has shown that if this temperature be exceeded serious errors are introduced owing, no doubt, to the accelerated caramelisation of carbohydrate material. However, by varying the time, it has been shown that the disturbing effect of the sugar increases with the period of contact, and that for a period of 2 hours, the time now recommended by Ritter *et al.* [1932], it is small though appreciable, as will be seen in Table V.

It is noteworthy that Dore [1920] many years ago recommended the use of the short time of contact with acid of 3½ hours.

Table V. *Effect of duration of acid treatment on the lignin yield of xylose added to hydrolysed oat straw (5 % acid for 1 hour).*

Varying times with 72 % acid, temp. <20°; diluted to 3 % and boiled for 2 hours.

0.5 g. straw + 0.5 g. xylose.

		Apparent lignin g.	Increase due to xylose and time mg.
Straw alone	16 hours	0.1105	—
" + xylose	1 hour	0.1178	7.3
" + "	2 hours	0.1172	6.7
" + "	4 "	0.1206	10.1
" + "	7 "	0.1380	27.5
" + "	16 "	0.1725	62.0
Straw alone	2 "	0.1111	—

A similar effect of the time of standing with 72 % acid may be observed with any material containing pentose, as for example a hardwood. In Table VI is given the apparent lignin content of Danzig oak at various periods.

Table VI. *Effect of duration of acid treatment on apparent lignin content of Danzig oak.*

Results expressed on original material, extracted with alcohol-benzene before use.
Varying times with 72 % acid, temp. <20°; diluted to 3 % and boiled for 2 hours.

Duration of acid treatment hours	Apparent lignin %
1	23.6
2	24.0
4	23.8
7	26.1
16	28.8

Further evidence of the fact that at 2 hours the disturbance due to pentose is small was obtained by adding a hemicellulose preparation to hydrolysed oat straw, and treating the mixture for only 2 hours with the 72 % acid. These results given in Table VII should be compared with Table IV in which the time of contact was 16 hours.

Table VII. *Addition of crude hemicellulose to hydrolysed oat straw (5 % acid, 1 hour).*

2 hours with 72 % acid, temp. <20°; diluted to 3 % and boiled for 2 hours.

	Apparent lignin g.	Lignin in hemi- cellulose g.	Apparent lignin from straw g.	Apparent lignin on straw %
0.5 g. straw alone	0.1277	—	0.1277	25.54
" " + 0.1 g. hemicellulose	0.1325	0.0043	0.1282	25.64
" " + 0.3 g. " "	0.1455	0.0128	0.1327	26.54
" " + 0.5 g. " "	0.1544	0.0213	0.1331	26.62
0.3 g. hemicellulose alone	0.0141	0.0128	—	—

(c) *Effect of removal of hydrolysable constituents.*

In the case of any normal plant material containing perhaps a considerable amount of xylan in the cellulosan fraction of the cellulose and xylose in the encrusting hemicelluloses also, the disturbance due to pentose may apparently be minimised or much reduced either by removing that fraction or by shortening the period of exposure to acid to 2 hours, as Ritter *et al.* [1932] have done, or by a combination of these two expedients. The hydrolysable constituents, amongst them the pentose-containing polysaccharides, have been progressively removed from a number of materials by mild acid treatment, and the apparent lignin obtained after standing for 16 hours with 72 % acid has been compared with that obtained after standing for only 2 hours. In the latter case boiling after dilution to 3 % was continued for 4 hours, as recommended by Ritter *et al.* [1932], though there does not seem to be any obvious advantage to be gained by so doing. It should be stated that, in general, the materials having the longer exposure to the strong acid, settled and filtered the more rapidly. The results are summarised in Table VIII. There is a steady fall in the apparent lignin content of these materials as the hydrolysable polysaccharides are progressively removed and, as would be expected, this fall is greater in magnitude when the longer period of contact is employed, for this gives rise to more apparent lignin

from the hydrolysable substances than the shorter contact. The figure on the untreated material at 2 hours is always considerably lower than that at 16 hours, but this difference disappears when the major part of the hydrolysable constituents has been removed, and after treatment for 1 hour with either 3 or 5 % acid, the figures are not significantly different whichever length of treatment is given.

Table VIII. *Comparison of 16-hour and 2-hour treatments of acid-hydrolysed materials.*

Results expressed as percentages of original material, extracted with alcohol-benzene before hydrolysis. All treatments given at the boil.

Treatment		Residue	Furfuraldehyde yield	Apparent lignin	
				16 hours	2 hours
Oat straw A:					
Untreated		—	Not determined	18.48	15.29
Water	1 hour	80.00	"	14.79	12.49
0.5 % H ₂ SO ₄	$\frac{1}{2}$ "	73.20	"	13.69	12.74
1.0 % "	1 "	61.26	"	12.24	12.75
5.0 % "	1 "	54.47	"	12.04	12.12
5.0 % "	5 hours	50.28	"	11.94	12.13
Beech wood:					
Untreated		—	"	23.31	21.47
Water	1 hour	94.08	"	22.09	20.70
0.5 % H ₂ SO ₄	$\frac{1}{2}$ "	92.04	"	21.99	20.27
1.0 % "	1 "	84.80	"	21.04	19.37
5.0 % "	1 "	70.57	"	19.59	19.23
5.0 % "	5 hours	65.15	"	18.68	19.05
Oat straw B:					
Untreated		—	17.88	16.22	14.82
Water	1 hour	80.05	15.81	13.94	13.41
0.5 % H ₂ SO ₄	$\frac{1}{2}$ "	72.69	12.46	13.53	13.02
1.0 % "	1 "	60.71	7.25	12.14	12.05
3.0 % "	1 "	56.56	6.98	11.54	11.52
5.0 % "	1 "	51.93	3.36	11.90	11.84
Oak (Danzig):					
Untreated		—	12.70	28.37	25.41
Water	1 hour	87.58	12.17	22.53	21.82
0.5 % H ₂ SO ₄	$\frac{1}{2}$ "	78.47	8.50	21.46	20.61
1.0 % "	1 "	76.74	8.17	22.25	20.95
3.0 % "	1 "	70.03	5.73	21.28	20.51
5.0 % "	1 "	67.22	4.68	21.01	20.59
Bean straw:					
Untreated		—	10.74	17.03	16.56
Water	1 hour	77.77	9.78	16.58	15.06
0.5 % H ₂ SO ₄	$\frac{1}{2}$ "	71.63	9.09	15.57	15.40
1.0 % "	1 "	64.65	7.87	14.42	14.34
3.0 % "	1 "	58.83	5.37	14.46	14.55
5.0 % "	1 "	56.80	5.28	15.54	14.35

Now, in their method, Ritter *et al.* [1932] employ an extraction with boiling water for 3 hours prior to treatment for 2 hours with the concentrated acid. The lower results obtained after this pretreatment they explain as being due to the removal of "extractives," insoluble in alcohol-benzene and insoluble in sulphuric acid or converted into insoluble products by the acid treatment. It seems however that a treatment for 3 hours with boiling water ought to be considered to be in part hydrolytic in action in addition to effecting extraction. They give no figures for the amount removed from the various woods by this treatment, but beyond doubt it is quite considerable and includes some encrusting hemicellulose.

The actual amount will vary widely from material to material according to the quantity and resistance of the hemicellulose constituents. In the five samples given in Table VIII, all of which were mature, boiling with water for 1 hour removed from 6 to 23 %. Table IX gives a comparison of the loss on treating several materials for 15 minutes and 3 hours with water at 100° and the furfuraldehyde yield of the material after the latter treatment.

Table IX. *Loss on treatment with water at 100°.*

Material	Furfuraldehyde yield %	Residue 15 mins. %	Residue 3 hrs. %	Furfuraldehyde yield on original basis %
Oak	15.2	94.3	92.3	14.4
Oat straw	17.6	80.4	77.2	14.2
Hay	12.1	72.4	69.9	11.1

The conclusion appears inevitable that the "extractives" removed by prolonged boiling of woods or mature plant materials are partly of a hemicellulosic nature and may contain a considerable portion of the "pentose not in cellulose."

The differences obtained by Ritter *et al.* [1932] between untreated and water-extracted woods may therefore be ascribed, at least in part, not to hypothetical "extractives" but to a pentose disturbance, the actual disturbance being comparatively small because the time of standing is only 2 hours. In Table V the increase in 2 hours produced by 0.5 g. xylose added to 0.5 g. hydrolysed straw was only 6 mg. or about 5 % of the lignin although the ratio of pentose to lignin present was about 5, a ratio which would never occur in mature materials but which might be found in slightly lignified materials. The conditions chosen by Ritter *et al.* [1932] involving prolonged water-extraction and brief acid treatment therefore are such that the pentose disturbance is quite small, but it would be incorrect to say that it does not occur or is negligible in all cases. In Table X are given some results bearing on this point, since it provides a comparison of the effects of the addition of pure xylan (prepared by the cold alkaline extraction of prepared oat straw cellulose) to straw untreated and to straw boiled for 3 hours with water.

Table X. *The addition of xylan to untreated and water-extracted oat straw.*

Various times with 72 % acid; temp. <20°; diluted to 3 % and boiled for 2 hours.

	Time in hours	Apparent lignin on straw %
0.8 g. straw boiled 3 hours (residue 0.611 g.)	2	13.36
0.8 g. straw + 0.5 g. xylan together boiled 3 hours (combined residue 0.632)	2	13.39
0.8 g. straw boiled 3 hours (residue 0.611 g.), then 0.5 g. xylan added	2	14.50
0.7 g. straw alone	2	16.63
" " + 0.3 g. xylan	2	16.77
" " alone	16	19.71
" " + 0.3 g. xylan	16	22.07

While untreated straw had an apparent lignin content of 16.63 % the water-extracted straw gave a figure of only 13.36 %. The addition of xylan to either produced only a slight increase. In the case of the untreated straw this was presumably because the pentose disturbance was already high, and as shown

in Table I there is no linear relationship, increasing amounts causing smaller and smaller increments in disturbing condensation products. In the case of the water-extracted mixture, on the other hand, the increase was slight because practically all the added xylan went into solution during the aqueous extraction, only 21 mg. out of the 0.5 g. originally present remaining. The xylan, as prepared, is not easily soluble in water but is apparently almost completely dissolved by this prolonged boiling, indicating again the hydrolytic effect of such a treatment. When, however, the xylan was added after the water-extraction of the straw, there was a considerable increase in apparent lignin even at 2 hours. It has been claimed that the Ritter *et al.* [1932] method is entirely unaffected by the presence of carbohydrates, but no direct or conclusive evidence is offered by them upon this important point. Nevertheless from this work it seems that the product obtained by their procedure will contain a minimum of condensation products and is certainly purer than that given under the best conditions by any other existing method employing 72 % acid. The question arises, however, as to whether it might not be improved and rendered more accurate by substituting an acid hydrolysis for the prolonged boiling with water, and thus rendering more certain and more complete the removal of encrusting hemicelluloses and celluloses which, containing pentose as they do, may cause the lignin figure to be too high, even with only 2 hours' contact with strong acid. The crucial point is whether lignin, as it exists in the plant material, is affected by treatment with hot dilute acids. The assumption might be made that it is resistant to boiling with 3 % H_2SO_4 , after treatment with 72 % acid, since this is normally a part of the lignin determination. Conceivably, however, before such treatment it may be more susceptible, and consequently to introduce such a pretreatment may result in lowering the lignin yield. At present there is no satisfactory evidence upon this point, and indeed, the planning of any conclusive experiments to decide the matter presents many difficulties. The fate of the methoxyl groups is not a satisfactory index of changes in the lignin, inasmuch as Ritter and Kurth [1933] by isolating a lignin-free "holocellulose" fraction (cellulose + hemicelluloses) showed that a portion of the methoxyl and most of the acetyl groups were associated with these polysaccharides rather than with the lignin. If there is any loss of lignin by pretreatment with dilute acid it would not appear to be large, for, as will be seen in Table VIII, there was no significant difference between materials treated with 3 % acid for 1 hour, 5 % acid for 1 hour, or subjected to the unnecessarily long treatment of 5 % acid for 5 hours, when the lignin was determined after standing for 2 hours with 72 % acid. With a 16-hour period, rather curiously, the last treatment gave lower figures. To sum up this series and many other pretreatments, it seems that for some materials a pretreatment for 1 hour with 1 % acid followed by a 2-hour treatment with the 72 % H_2SO_4 is sufficient to give minimum results, and for others, particularly straws, pretreatment with rather stronger acids is necessary. If 3 or 5 % acid be employed it is of little moment whether the duration of contact with 72 % acid be 2 or 16 hours.

From information obtained from the U.S. Forest Products Laboratory it is suggested, not that lignin is affected by the mild acid hydrolysis, but that there is present a small amount of some product intermediate between carbohydrate and lignin which is acid-soluble or hydrolysable, but which is further condensed by the strong acid treatment, so that it then becomes resistant to dilute acid. Further, since this product is rendered soluble, like lignin, by chlorination, it should properly be regarded as lignin, and therefore acid pretreatment methods which remove it are inadmissible. This is an interesting alternative suggestion,

but as yet there does not seem to be any experimental evidence directly supporting it. The validity of an acid pretreatment must therefore remain undecided until the question of the action of dilute acids on lignin has been settled. Investigations along this line are proceeding.

(d) *The lignin content of various plant materials.*

In view of the observations recorded above, it is clear that the recorded figures for lignin content of all classes of plant materials are too high to a greater or less extent, with the exception of those few determined since 1932 by the method of Ritter *et al.* [1932]. The error will vary considerably according to the pentose content of the material in question and the temperature and time of treatment with 72 % acid. It has frequently been observed that analyses add up to more than 100 % and this may be ascribed almost always to the excessive lignin figure. Phillips [1934] has recently criticised the indiscriminate application of methods developed for woods to other classes of plant materials, but in so far as mature materials are concerned, it has been shown herein that the errors are inherent in the method itself and not caused by any wide difference in composition between woods and "non-woods." Table XI contains analyses made on a very wide range of woods and plant materials, the lignin figure being recorded before and after extraction for 1 hour with 5 % H_2SO_4 . The final column contains the percentage reduction in apparent lignin produced by this treatment, which may amount to one-quarter or even one-third in special cases. As might be expected from their higher pentose contents, the reduction in the case of some hard woods is greater than that for soft woods. It will be noted

Table XI. *The lignin content of various materials before and after extraction with 5 % H_2SO_4 for 1 hour.*

16 hours with 72 % acid, temp. $<20^\circ$; diluted to 3 % and boiled for 2 hours.
All results expressed on 100 g. original material before alcohol-benzene extraction.

Material	Apparent lignin %	Loss of original material on hydrolysis %	Lignin after hydrolysis %	Reduction in apparent lignin figure %
Oak	28.80	34.41	20.24	29.9
Beech	23.30	29.43	19.50	16.3
Basswood	22.82	27.92	17.98	21.1
Teak	28.98	34.42	26.95	7.2
Mahogany	27.91	23.38	25.77	7.5
African walnut	32.87	18.39	30.33	7.9
Deal	25.87	26.81	23.42	9.6
Sitka spruce	26.18	22.10	23.46	10.3
Pine needles	26.85	51.49	23.91	11.2
Almond hulls	30.49	55.93	22.21	27.2
Banana leaves	19.67	41.99	16.72	15.2
Coconut husk	40.86	28.09	34.66	15.2
Moss	21.99	50.19	16.21	26.3
Flax straw	24.94	30.37	21.79	12.4
Oat straw	18.48	45.53	12.04	34.8
Wheat straw	19.31	38.72	13.90	28.0
Barley straw	16.70	52.13	10.53	37.1
Mature hay	18.50	46.26	14.10	23.8
Bracken	30.28	54.65	23.67	21.8
Mustard plants	12.41	59.59	9.43	24.0
Maize plants	11.14	57.72	7.81	29.9
Barley plants	6.13	68.12	3.78	38.4
Lucerne tops	10.93	68.01	8.20	24.8
Lucerne roots	11.49	58.95	8.67	24.8

that all but the last five materials are mature "lignified" materials; the remaining samples were younger and considerably higher in nitrogen. For reasons which will appear in the next paper these samples are subject to an additional and different error, but the figures are quoted for comparison.

In a few samples the lignin content was determined also by the full Ritter *et al.* [1932] method and these results are given in Table XII for comparison with Table XI. The results fall midway between the apparent lignin at 16 hours and that obtained after a hydrolytic pretreatment.

Table XII. *The lignin content of various materials by the Ritter, Seborg and Mitchell method.*

2 hours with 72 % acid, temp. < 20°; diluted to 3 % and boiled for 4 hours.
All results expressed on 100 g. original material.

	Lignin %		Lignin %
Basswood	20.44	Sitka spruce	25.38
Teak	27.10	Flax straw	23.14
Deal	25.78	Wheat straw	16.62

(e) *Implications of the observations recorded above.*

In two directions at least there are to be found implications of the data presented. The first lies in the preparation of lignin in bulk for ultimate analysis, constitutional studies or the preparation of derivatives. If the sulphuric acid method of preparation be employed, as it is by a number of workers, precautions must be taken to avoid the formation of condensation products from pentose groupings which, under bad conditions, might amount to 25-30 % of the preparation. The presence of such a fraction would have a serious effect on the figures for ultimate analysis, and might interfere with chemical reactions. Sherrard and Harris [1932] have studied the conditions of preparation of lignin from sugar maple and have introduced various modifications which they claim yield lignin free from carbohydrate. It is by no means certain, however, that the product is entirely free from these disturbing condensation products, for the acid treatment recommended is for a period of 15 hours from the time when the material goes into a thin solution, and no pretreatment other than an aqueous extraction is carried out. To avoid any possibility of the presence of such a fraction, a pretreatment with dilute acid would be advisable and the shortening of the period of contact with 72 % acid to 2 or 3 hours. A light friable product may be obtained by this means.

A second important application of these experiments lies in the studies of microbiological decomposition of plant materials, and in digestibility trials, in both of which lignin determinations may be carried out on materials which are changing as regards their other constituents. In the decomposition of such a material the hemicelluloses are rapidly fermented away, and the consequent fall in pentose groupings may produce an accompanying fall in the apparent lignin content. The effect therefore resembles that produced by progressive acid hydrolysis, as in Table VIII, in which the apparent lignin content falls without there being necessarily any loss of true lignin. Unless precautions are taken to minimise the pentose disturbance, there may appear to be a wholly illusory loss of lignin. Various conclusions as to the decomposibility of lignin require confirmation for this reason. Some experiments on this point are in progress.

(f) *The nature of the disturbing material.*

Throughout this paper, the substance formed from pentose in the presence of 72 % sulphuric acid has been referred to vaguely as a condensation product. Some evidence has been obtained which points to the view that the condensation is between the lignin molecule and furfuraldehyde, the latter being produced from pentose by dehydration. That furfuraldehyde will combine with phenolic bodies is well known, indeed its determination ordinarily depends on the production of an insoluble phloroglucide with phloroglucinol. Insoluble "resins" have been manufactured commercially by the condensation of furfuraldehyde with higher phenols. Since there is general agreement that the lignin molecule contains a phenolic grouping, and since Ross and Hill [1929] have shown that lignin undergoes a definite reaction with formaldehyde, it seems not unreasonable to suppose that furfuraldehyde also might condense with lignin. It was later found that the condensation of furfuraldehyde with isolated alkali lignins had been the subject of a patent by Phillips [1930]. The possibility of condensation *in situ* was confirmed by the addition of increments of redistilled furfuraldehyde to a lignin determination on straw, and the results are given in Table XIII. The furfuraldehyde was added half an hour after the straw had gone into solution. The precipitates obtained after dilution and boiling were very black, flocculated well and settled rapidly.

Table XIII. *The addition of furfuraldehyde to lignin determinations.*

16 hours with 72 % H_2SO_4 , temp. $<20^\circ$; diluted to 3 % and boiled for 2 hours.

Material	Apparent lignin content g.	Increase per 0.025 ml. addition g.
0.8 g. straw alone	0.1587	—
" " + 0.025 ml. furfuraldehyde	0.1943	0.0356
" " + 0.050 ml. "	0.2248	0.0305
" " + 0.075 ml. "	0.2617	0.0369
" " + 0.100 ml. "	0.2942	0.0325
" " + 0.200 ml. "	0.3738	0.0199

Average 0.034

The results show that there is an apparent increase even larger than the quantity of furfuraldehyde added (sp. gr. 1.16). Up to the addition of 0.1 ml. of furfuraldehyde the increase is approximately proportional to the amount added, the pipetting of such small quantities not being very accurate. Above 0.1 ml. further additions did not give so large an increase. Controls of the same quantities of furfuraldehyde treated alone yielded no weighable precipitate, though there was the formation of a floating film with 0.2 ml. Larger quantities, however, gave a black granular precipitate, for example, 1 ml. with 15 ml. 72 % acid, yielded 46 mg. of precipitate after dilution and boiling. Table XIII shows that for each addition of 0.025 ml. furfuraldehyde there was an average increment in precipitate of 0.034 mg., a clear indication of a linkage between the aldehyde and lignin and some further reaction the nature of which is not understood.

To support the view that the pentose disturbance is due to furfuraldehyde production and subsequent condensation, the presence of furfuraldehyde was detected and the amount determined in experiments in which xylose was treated with 72 % sulphuric acid alone. After standing and dilution to 3 % the solution was steam-distilled, 300 ml. distillate being collected from 600 ml. solution. It is not known whether under these conditions the recovery is quantitative. To the distillate were added 150 ml. concentrated HCl to bring the acid concn-

tration to 12 %. Phloroglucinol was added and the precipitate weighed after standing overnight. The residue after steam-distillation was boiled for a further period of 1½ hours and filtered.

Table XIV. *Furfuraldehyde yield from xylose and arabinose with 72 % H_2SO_4 .*

	Time with 72 % acid hours	Phloroglucide mg.	Furfuraldehyde mg.*	Residue mg.
1 g. xylose	16	19.9	13.0	39.7
" "	11	12.1	8.9	11.6
" "	7	9.9	7.8	10.0
" "	4	16.0	10.9	5.9
" "	2	5.0	5.3	None
" "	24	37.1	21.9	49.4
" arabinose	16	5.1	5.3	4.1

* Calculated from the formula $(a + 0.0052) \times 0.517$.

The amount of furfuraldehyde present seems to change with time, presumably by transformation into insoluble residue. The nature of this residue is unknown, but it is possibly a compound formed by slow dehydration and condensation of the furfuraldehyde. Meunier [1929] has stated that furfuraldehyde may condense with loss of water to give brown derivatives.

From these experiments it seems that the insoluble residue formed from xylose alone in the presence of 72 % sulphuric acid may have no part in the increase in apparent lignin caused by xylose when added to straw. That this might be the case was suspected earlier, since the increase found is always so much greater than would be expected from the residue obtained from xylose alone.

The probability is therefore that the disturbance caused by pentose is due to the production of furfuraldehyde and the more or less rapid condensation of this with the lignin present, to give an insoluble phenol-furfuran "resin," which is very stable and from which the furfuraldehyde cannot readily be split off. In the event of there being present furfuraldehyde in excess of the combining power of the lignin, dehydration of the former might occur with the formation of another insoluble precipitate. This however is an unlikely occurrence inasmuch as the amount of lignin usually present in mature materials could combine with far more aldehyde than would be produced from the pentose there, and the first reaction therefore takes precedence.

The observed difference between the effects of addition of xylose and arabinose may be explained by the fact, already known, that the production of furfuraldehyde from these two sugars takes place at different rates, and that the theoretical equation does not adequately represent the changes taking place. Since the uronic acids may be dehydrated to yield furfuraldehyde it is possible that they too might interfere in this way in the lignin estimation. This has not been verified. The disturbance due to fructose and consequently to sucrose may also provisionally be ascribed to the production of hydroxymethylfurfuraldehyde or possibly to β -hydroxy- γ -methylfurfuraldehyde which is produced from fructose under certain conditions of dehydration.

SUMMARY.

1. Some of the disturbing factors concerned in the determination of lignin by the 72 % sulphuric acid method have been investigated, and their effects in various recommended procedures have been studied.

2. Certain sugars, particularly xylose and fructose, give an insoluble residue on standing with 72 % sulphuric acid and increase the apparent lignin figure when added to plant materials. Arabinose does so also, to a slight extent, and sucrose, by reason of its fructose constituent. Polysaccharides containing pentose sugars produce a similar effect.

3. The disturbance caused by the presence of such carbohydrates increases with the time of contact with 72 % acid. At 2 hours the effect is small.

4. In plant materials the effect of the presence of xylose in the hemicellulose may be almost excluded by a hydrolytic pretreatment with dilute mineral acids, or minimised by reducing the time of contact to 2 hours, as in the Ritter-Seborg-Mitchell method. The validity of an acid pretreatment is not proved, since the action of dilute acids on lignin is not known.

5. Because of the presence of xylose, the figures generally quoted for lignin are, in most cases, too high. The lignin content determined after acid pretreatment is given for a wide range of plant materials and woods.

6. The disturbance due to pentose is caused by the slow production of furfuraldehyde and its condensation with lignin to form an insoluble phenol-furfuran resin. Furfuraldehyde itself may give an insoluble product by dehydration and condensation, but the former reaction probably takes precedence as long as there are phenolic groups on the lignin unsatisfied.

REFERENCES.

- Cohen and Dadswell (1931). *U.S.I.R. Australia, Division of Forest Products, Technical Paper*, No. 3.
Dore (1920). *Ind. Eng. Chem.* **12**, 476, 984.
Hilpert and Littman (1934). *Ber. deutsch. chem. Ges.* **67**, 1551.
Meunier (1929). *Chim. Ind.*, Special No., 553.
Norman and Jenkins (1933). *Biochem. J.* **27**, 818.
Ost and Wilkening (1910). *Chem. Ztg.* **34**, 461.
Peterson, Walde and Hixon (1932). *Ind. Eng. Chem., Anal. Edit.* **4**, 216.
Phillips (1930). U.S. Pat., 1750903.
—— (1932). *J. Assoc. Off. Agric. Chem.* **15**, 118.
—— (1934). *J. Assoc. Off. Agric. Chem.* **17**, 277.
Ritter and Kurth (1933). *Ind. Eng. Chem.* **25**, 1250.
—— Seborg and Mitchell (1932). *Ind. Eng. Chem., Anal. Edit.* **4**, 202.
Ross and Hill (1929). *Pulp and Paper Mag. Canada*, **27**, 541.
Schwalbe (1925). *Papier-Fabr.* **23**, 174.
Sherrard and Harris (1932). *Ind. Eng. Chem.* **24**, 103.
Willstätter and Zechmeister (1913). *Ber. deutsch. chem. Ges.* **46**, 2401.

[FROM THE BIOCHEMICAL JOURNAL, Vol. XXVIII, No. 6, pp. 2160–2168, 1934]

[*All Rights reserved*]

CCLXXXIV. THE DETERMINATION OF LIGNIN.

II. ERRORS INTRODUCED BY THE PRESENCE OF PROTEINS.

By ARTHUR GEOFFREY NORMAN
AND SAMUEL HARRY JENKINS.

*From the Biochemistry Section and Fermentation Department,
Rothamsted Experimental Station.*

(Received October 29th, 1934.)

THE methods for the determination of lignin have mainly been developed by wood chemists upon materials which are very low in nitrogenous constituents. When however it is desired to apply these methods to less mature plant materials, containing considerable amounts of protein, it has been found that the isolated lignin residue contains some nitrogen. Paloheimo [1925] recognising this stated that all lignin figures obtained on nitrogenous materials should be corrected by subtracting an amount equal to the nitrogen content of the lignin $\times 6.25$. In other words he made the assumption that the nitrogen in the lignin residue is present as protein. The same correction has been employed by Waksman and Stevens [1930] and by Phillips [1932]. The latter worker, however, admits that this may not be justified but considers that at present it is the best procedure that can be adopted.

The experiments to be described deal with the effect of the presence of protein on the lignin figure as determined by 72 % acid treatment and were designed partly to test the validity of such a correction and partly to seek such conditions as should minimise the disturbance from this source.

EXPERIMENTAL.

(a) The nature and magnitude of the disturbance due to protein.

If proteins, such as egg-albumin or caseinogen, in a finely divided condition are allowed to stand for 16 hours with 72 % H_2SO_4 , and the solution is then diluted to 3 % and boiled, no precipitate is obtained; if they are added to pure cellulose, such as filter-paper, and similarly treated, the precipitate, if any, is negligible. On the other hand, if protein be added to straw and the mixture treated as above, the apparent lignin content of the straw will be increased and the lignin residue will contain some nitrogen. In effect, therefore, the lignin itself, or some other straw constituent, acts as a precipitant for a portion of the nitrogenous material in solution. This may be seen from Table I. All lignin determinations in this and subsequent Tables, unless a statement to the contrary is made, were carried out in quadruplicate, two then being used for the ash correction and two for the estimation of nitrogen.

All determinations made in the presence of added protein filtered more slowly, the rate being roughly proportional to the amount added. The effect of the

Table I. *The addition of caseinogen to oat straw and hydrolysed oat straw (5 % acid, 1 hour).*

Straw 0.52 % N. Hydrolysed straw 0.47 % N. Caseinogen 12.43 % N.
16 hours with 72 % acid, temp; <20°; diluted to 3 % and boiled for 2 hours.

Mixture	Apparent lignin g.	N in lignin mg.	N in straw mg.	N in casein- ogen mg.	Total N present mg.	% N re- covered	Increment in lignin g.	Increment in lignin N in lignin
(A) 0.8 g. straw alone	0.1449	2.33	4.16	—	4.16	56	—	—
" " +0.2 g. caseinogen	0.2375	4.25	4.16	24.85	29.01	15	0.0826	43.0
(B) 0.4 g. hydroly. straw alone	0.1059	1.34	1.88	—	1.83	71	—	—
" " +0.2 g. caseinogen	0.1271	5.06	1.88	24.85	26.73	19	0.0222	6.0

presence of protein seemed to be to give a cloudy solution in which the lignin did not settle out but remained partly in suspension. Mixtures containing a high percentage of protein could not be filtered at all. In Table I is given a balance sheet of the nitrogen present initially and recovered in the lignin. Only 15 % remained when 0.2 g. caseinogen was added to the straw and 19 % when it was added to a smaller, but approximately equivalent, amount of hydrolysed straw. In order to throw some light on the factor which should be applied to correct for this nitrogen disturbance, the ratio of the increment produced by the addition of protein to the increase in nitrogen found in the lignin has been calculated. This was widely divergent in the two cases, being much higher in the case of the untreated straw. This suggested the possibility of some interaction between hydrolysable constituents, perhaps pentose, and protein, as a result of which the disturbing effect of both might be mutually enhanced. To test this point a number of mixtures of xylose and caseinogen were set up in quadruplicate and treated as in the lignin determination.

Table II. *Interaction of xylose and protein in production of apparent lignin.*

16 hours with 72 % H₂SO₄, temp. <20°; diluted to 3 % acid and boiled for 2 hours.

Mixture	Apparent lignin g.	N in lignin mg.	N in caseinogen mg.	% N recovered	Increment due to caseinogen g.	Increment N in lignin
0.5 g. xylose alone	0.0078	—	—	—	—	—
" " +0.01 g. caseinogen	0.0227	0.6	1.21	48	0.0149	24.8
" " +0.025 g. "	0.0219	1.5	3.11	48	0.0171	11.4
" " +0.05 g. "	0.0181	1.25	6.22	20	0.0103	8.2
" " +0.1 g. "	0.0339	5.0	12.43	40	0.0261	5.2
" " +0.15 g. "	0.0231	1.4	18.65	7	0.0153	10.9
" " +0.2 g. "	0.0026	0.25	24.86	1	—	—
" " +0.25 g. "	0.0003	—	31.08	—	—	—

That there is an interaction between pentose and protein is clear, but its nature is rather obscure. At low proportions of protein to pentose the effect is markedly to increase the production of insoluble material from the pentose, so much so that when the rate of xylose to caseinogen was highest the apparent lignin figure was almost trebled and nearly 50 % of the added nitrogen was recovered. The latter is not in proportion to the increment caused, which amounted to more than the caseinogen added. With larger additions of protein, however, the yields of insoluble residue decrease, even below that which would be given by the xylose alone, until the point is reached when there is virtually no precipitate at all. Thus the result of the interaction between pentose and protein seems to be much to enhance the effect of the former at low concen-

tration, of the latter, but at high concentrations to reduce or even to nullify it. The nature of the reaction involved has not been investigated, but no doubt it is concerned with the production of furfuraldehyde from the xylose and its linkage with amino-groups.

This variable interaction makes it extremely difficult, if not impossible, to predict the magnitude of the disturbance to be expected in any particular material. That the same order of diminishing disturbance with increasing protein takes place with natural materials is seen in the first part (A) of Table III. Unfortunately it was not possible to test the effect of a further addition of caseinogen as the filtration was then impossibly slow. With 0.1 g. caseinogen added, the lignin required 1 hour for filtration, with 0.2 g., 1 day, and with 0.3 g., 5 days.

The main purpose of the experiments summarised in Table III was to attempt to distinguish between the protein disturbance alone, that produced by pentose and the interaction of the two. For this purpose the amount of hydrolysed straw taken was exactly equivalent to the untreated straw, it being determined that the loss on hydrolysis was 44.91 %. Inasmuch as in hydrolysed material the pentose content is quite low (the furfuraldehyde yield was reduced by about 85 % by the treatment), the figures in the second part (B) of Table III may be taken to represent the effect of protein alone. The magnitude of the

Table III. *The effect of addition of caseinogen to oat straw and to an equivalent amount of hydrolysed oat straw (5 % acid, 1 hour).*

Conditions as in Table I.									
Mixture		Apparent lignin g.	N in lignin mg.	N in straw mg.	N in casein- ogen mg.	Total N present mg.	% N re- covered	Incre- ment due to casein- ogen	Increment of N in lignin
(A)	0.571 g. straw alone	0.1059	1.65	2.97	—	2.97	55	—	—
	“ “ +0.1 g. caseinogen	0.1245	4.5	2.97	12.43	15.40	29	0.0186	6.5
	“ “ +0.2 g. “	0.1361	5.65	2.97	24.85	27.82	20	0.0312	7.8
	“ “ +0.3 g. “	0.1264	5.6	2.97	37.28	40.25	14	0.0205	5.2
(B)	0.315 g. hydroly. straw alone	0.0696	0.9	1.48	—	1.48	61	—	—
	“ “ +0.1 g. caseinogen	0.1113	4.1	1.48	12.43	13.91	29	0.0417	13.0
	“ “ +0.2 g. “	0.1107	4.75	1.48	24.85	26.33	18	0.0411	10.7
	“ “ +0.3 g. “	0.1136	4.45	1.48	37.28	38.76	12	0.0440	12.4
(C)	0.315 g. hydroly. straw +0.2 g. xylose	0.0916	1.15	1.48	—	1.48	78	—	—
	“ “ +0.2 g. xylose	0.1247	5.1	1.48	12.43	13.91	37	0.0331	8.4
	+0.1 g. caseinogen								
	0.315 g. hydroly. straw +0.2 g. xylose	0.1278	5.7	1.48	24.85	26.33	22	0.0362	8.0
	+0.2 g. caseinogen								
	0.315 g. hydroly. straw +0.2 g. xylose	0.1251	5.5	1.48	37.28	38.76	14	0.0335	7.7
	+0.3 g. caseinogen								

increment produced does not change greatly with 0.1, 0.2, or 0.3 g. of added caseinogen, and as a result the percentage of nitrogen recovered in the lignin decreases. The ratio of increment produced to increase in nitrogen found is higher than it would be if the increment were merely protein in nature. This is in contrast to the rather similar conditions of part (B) in Table I in which the ratio is about 6.0. The conclusion to be drawn from both these experiments is, however, that there is some interaction between protein and lignin in the presence of 72 % acid as a result of which an insoluble product is formed. That combination between lignin and protein can take place has been shown by Hobson and Page [1932] and by Waksman and Iyer [1932]. The product obtained by them is stable and the nitrogen cannot easily be removed. It seems not improbable that the disturbance caused by protein in the lignin determina-

tion is due to the formation of some compound of a similar sort. This combination cannot however be a direct one or else the ratio of the increment produced over nitrogen present would approximate to 6. Because of the variability of this ratio, the assumption is that it is protein degradation products formed by the action of the strong acid, rather than protein itself, which are concerned. The fact that in Table III (B) the increments in apparent lignin did not increase with increasing additions of protein rather suggests that the lignin has a limited combining power with such nitrogenous products, and that this was fully satisfied by the lowest amount added.

Turning now to Table III (A) in which untreated straw was used, three factors are involved, firstly the disturbance due to pentose, secondly that due to protein and thirdly that due to the interaction between pentose and protein, which, as shown earlier, might result in a reduction of the effect of the first factor. The increments due to the addition of caseinogen are not nearly so great as in (B) when the equivalent amount of hydrolysed straw was used, and therefore, though the percentages of nitrogen recovered in the lignin are almost identical, the ratios of the increment over increase of nitrogen recovered are much lower. In this case the figures are of the same order as would be obtained if the increment were solely due to protein, but in view of other experiments this must be accounted a coincidence.

In Table III (C) the same three interacting factors are concerned as in (A), since by addition of xyllose to the hydrolysed material the effect of pentose and its interaction with protein are re-introduced. The yields of apparent lignin and the percentage recovery of nitrogen are all very similar to those in (A), as might be expected. The same sparing effect on addition of 0.3 g. caseinogen occurs. The close coincidence of these results on synthetic mixtures with those in part (A) may be taken as evidence that the factors concerned have been correctly distinguished.

In all these experiments described above the percentage of nitrogen recovered in the lignin is highest when small quantities of protein are present. Furthermore, increasing the protein beyond a certain point results actually in a decrease in the amount of apparent lignin formed. This is well illustrated in Table III (A) and (C) when the addition of 0.3 g. caseinogen in both cases gave a lower yield of apparent lignin than 0.2 g. That this fact is probably connected with the presence of pentose is shown by part (B) in which the same sparing effect

Table IV. *The effect of addition of small quantities of caseinogen to oat straw and to an equivalent amount of hydrolysed straw (5% acid, 1 hour).*

Conditions as in Table I.							Increment due to caseinogen g.	Increment in lignin of N in lignin
Mixture	Apparent lignin g.	N in lignin mg.	N in straw mg.	N in caseinogen mg.	Total N present mg.	% N recovered		
(A) 0.8 g. straw alone	0.1587	2.0	3.04	—	3.04	67	—	—
" " + 25 mg. caseinogen	0.1670	3.2	3.04	3.11	6.15	52	0.0083	6.9
" " + 50 mg. "	0.1817	4.6	3.04	6.22	9.28	49	0.0230	8.8
" " + 75 mg. "	0.1805	5.3	3.04	9.33	12.37	43	0.0218	6.6
" " + 100 mg. "	0.1765	5.2	3.04	12.43	15.47	34	0.0178	5.6
" " + 150 mg. "	0.1876	6.1	3.04	18.65	21.69	28	0.0289	7.0
(B) 0.415 g. hydroly. straw alone	0.1059	1.4	1.62	—	1.62	80	—	—
" " + 25 mg. caseinogen	0.1183	2.45	1.62	3.11	4.73	52	0.0124	11.8
" " + 50 mg. "	0.1230	3.0	1.62	6.22	7.84	38	0.0171	10.7
" " + 75 mg. "	0.1196	3.25	1.62	9.33	10.95	30	0.0137	7.4
" " + 100 mg. "	0.1177	3.2	1.62	12.43	14.05	23	0.0118	6.6
" " + 150 mg. "	0.1112	3.6	1.62	18.65	20.27	18	0.0053	2.4

was not manifest. Some experiments, similar in nature to those described in Table III, were made in which the effect of quite small additions of protein was studied, from 25 to 150 mg. caseinogen being added to 0.8 g. straw and to an equivalent amount of hydrolysed straw. These results are given in Table IV.

In (A) in which caseinogen was added to straw the percentage of nitrogen recovered in the lignin falls steadily, but the apparent lignin yield shows an initial rise, a secondary fall and then a further increase. The reason for this peak at the point when 50 mg. of caseinogen were present is not clear but, inasmuch as it occurs also in (B) in which hydrolysed straw is employed, it is probably connected with the interaction between lignin and protein, rather than a protein-pentose effect. The ratios of increment in lignin to the increase of nitrogen recovered are very variable in (A), all but one being significantly higher than 6, in contrast to (B) in which they fall steadily from 12 to 2.4. With an equivalent amount of hydrolysed straw and increasing amounts of caseinogen the percentage of nitrogen recovered in the lignin falls faster than in (A), demonstrating again that with smaller quantities of protein present there is a pentose-protein reaction resulting in the retention of a part of the nitrogen in an insoluble precipitate. The nature of this reaction is not known, but in view of the observations in the previous paper it is likely that furfuraldehyde is concerned in a condensation with some protein fission product or products.

The addition of a small quantity of furfuraldehyde to caseinogen in the presence of 72 % sulphuric acid resulted in a dark-coloured solution, the intensity varying with the amount of protein present. Only a trace of precipitate was formed on dilution and boiling, but it is perhaps significant that this was greatest in amount when the protein added was low (Table V).

Table V. *The addition of furfuraldehyde to small quantities of caseinogen.*

Conditions as in Table I.	
Mixture 0.25 ml. furfuraldehyde with	Weight of precipitate mg.
25 mg. caseinogen	4.2
50 mg. "	5.4
75 mg. "	4.1
100 mg. "	3.3
150 mg. "	2.5
200 mg. "	1.6

The most practical point arising out of these experiments is the fact that any attempt to calculate the disturbance due to protein and to apply a correction for it is useless in view of the interplay of the various factors involved.

(b) *The reduction of the disturbance due to protein.*

Since there appears to be no possible means of calculating from the nitrogen content of the lignin the error due to protein, means were sought by which it could be minimised. The first step was to examine whether the reduction of the time of contact with 72 % acid from 16 hours to 2 hours would effect any reduction in the nitrogen content of the lignin. Employing tares, rich in protein, the comparative figures obtained were:

1 g. tares:	16 hours.	Apparent lignin 0.1647 g. containing 9.25 mg. N.
1 g. tares:	2 hours.	Apparent lignin 0.1500 g. containing 9.9 mg. N.
1 g. lucerne:	16 hours.	Apparent lignin 0.1503 g. containing 6.9 mg. N.
1 g. lucerne:	2 hours.	Apparent lignin 0.1500 g. containing 6.3 mg. N.

Similar results were obtained with artificial mixtures of straw and protein, some of the figures being given in Table VI, from which it may be seen that there is relatively little difference in the percentages of nitrogen retained by the lignin.

Table VI. *Effect of time of contact with 72 % acid on the nitrogen in lignin from mixtures of straw and protein (egg-albumin).*

Time with 72 % acid stated, temp. <20°; diluted to 3 % and boiled for 2 hours.

Mixture	Time hours	Apparent lignin g.	N in lignin mg.	N in straw mg.	N in protein mg.	Total N present mg.	% N re- covered	Incre- ment due to protein g.	Increment N in ligni
1 g. straw alone	16	0.1848	2.45	5.2	—	5.2	47	—	—
" "	2	0.1529	2.0	5.2	—	5.2	38	—	—
1 g. straw + 0.1 g. protein	16	0.2275	6.65	5.2	11.71	16.91	39	0.0427	10.2
" "	2	0.2246	6.1	5.2	11.71	16.91	36	0.0717	17.5
1 g. straw + 0.25 g. protein	16	0.2482	9.5	5.2	29.27	34.47	27	0.0634	9.0
" "	2	0.2006	7.55	5.2	29.27	34.47	22	0.0477	8.6
0.6 g. hydrol. straw alone	16	0.1327	1.7	2.8	—	2.8	61	—	—
" "	2	0.1334	1.75	2.8	—	2.8	62	—	—
0.6 g. hydrol. straw + 0.25 g. protein	16	0.1638	6.2	2.8	29.27	32.07	19	0.0311	6.9
" " "	2	0.1760	6.75	2.8	29.27	32.07	21	0.0426	8.5

These and many other observations make it clear that the shorter period has no advantage as far as the nitrogen content of the product is concerned. To lower this, recourse must be had apparently to some pretreatment which reduces the nitrogen content of the original material without affecting the lignin itself. Even so, unless the removal of nitrogenous constituents be very complete little would be gained, since, as seen repeatedly in the previous section, the lignin disturbance is highest when small quantities of protein are present.

Attempts were made to remove the protein by a pepsin digest, alone, and followed by boiling for 1 hour with 5 % H_2SO_4 . 1 g. tares was suspended in 25 ml. 0.1 *N* HCl and, after the addition of 2 ml. of a 5 % pepsin solution, kept at 40°. The yields of lignin and its nitrogen content after this pretreatment are given in Table VII.

Table VII. *Attempted removal of protein by pepsin and acid hydrolysis.*

16 hours with 72 % H_2SO_4 , temp. <20°; diluted to 3 % and boiled for 2 hours.

1 g. material treated in various ways	Residue g.	Apparent lignin g.	N in lignin mg.
Untreated	1.000	0.1647	9.25
Digested with pepsin—3 hours	0.640	0.1391	6.35
Digested with pepsin—40 hours	0.566	0.1187	4.5
Digested with pepsin—3 hours, then hydrolysed 1 hour with 5 % acid	0.388	0.1122	4.5
Digested with pepsin—40 hours, then hydrolysed 1 hour with 5 % acid	0.368	0.1021	3.35
Hydrolysed 1 hour with 5 % acid	0.400	0.1177	5.4

Though the disturbance due to protein is much lowered by pepsin digestion, it is not markedly less than when a simple acid hydrolysis alone is given. Any biological treatment to be adopted, because of the time consumed, must show very definite advantages over a chemical treatment. Neither pepsin digestion nor fermentation with highly active protein-decomposing organisms, as tried subsequently, fulfilled this requirement. Of possible chemical treatments, alka-

line extraction, which would be the most effective, is ruled out because of its solvent action on lignin. In Table VIII is given the result of removing nitrogen from lucerne by various means.

Table VIII. *Removal of nitrogen from lucerne*
(original $N = 2.75\%$).

Nitrogen expressed as percentage of the original N content.

Treatment	% N recovered
Water at 100° — $\frac{1}{2}$ hour	60.2
NaCl 10 % at 40° —2 hours	48.7
H_2SO_4 5 % at 100° —1 hour	19.7
H_2SO_4 5 % at 100° —5 hours	13.1
Pepsin at 40° —3 days	19.0
Takadiastase at 40° —3 days	62.5

The most suitable treatment is acid hydrolysis for 1 hour with 5 % H_2SO_4 , the same procedure as was employed in previous work for the reduction of the disturbance due to pentose. It is still subject to the criticism that it has not been unquestionably shown that lignin is unaffected by dilute acid prior to contact with 72 % H_2SO_4 . Further, since the acid pretreatment does not completely remove protein, but only results in a lowering of the protein content, this expedient is not particularly satisfactory in the case of materials originally high in nitrogen. Indeed one or two cases have occurred in which the nitrogen in the lignin has been a little higher after such hydrolytic pretreatment. Examples of some green materials are given in Table IX.

Table IX. *Effect of hydrolytic pretreatment in lowering the nitrogen in lignin from certain green materials.*

All results expressed on 1 g. original material.

Material	N in material mg.	Apparent lignin mg.	N in lignin mg.	Recovery of N %
Kale 1 g.	42.8	53.5	2.0	5
Hydrolysed kale 0.279 g.	3.8	58.2	2.0	53
Maize 1 g.	27.6	182.4	9.6	35
Hydrolysed maize 0.365 g.	7.7	79.9	3.6	47
Bean 1 g.	34.2	150.2	5.2	15
Hydrolysed bean 0.372 g.	9.1	117.1	5.4	60
Grass 1 g.	32.4	179.3	10.3	32
Hydrolysed grass 0.414 g.	12.2	132.9	6.5	53
Wheat 1 g.	13.1	213.0	5.7	44
Hydrolysed wheat 0.504 g.	4.9	128.0	2.4	49

In two out of the five materials taken, the nitrogen in the lignin was almost unaffected by the hydrolytic pretreatment, while in the remaining three cases a considerable reduction was effected.

It was shown earlier that a shorter period of contact with the 72 % sulphuric acid did not result in any lowering of the nitrogen in lignin. This is also the case if the full procedure of Ritter *et al.* [1932] be used. Comparisons of this method with direct determinations are given in Table X.

Hydrolytic pretreatment, therefore, appears to be only partially successful

Table X. *Comparison of nitrogen in lignin obtained by Ritter-Seborg-Mitchell method with direct determinations.*

All results expressed on a basis of 1 g. original material.

Material	N content mg.	16 hours with acid		2 hours with acid		R.-S.-M. method		16 hours (after hydrolysis)	
		Apparent lignin g.	N in lignin mg.	Apparent lignin g.	N in lignin mg.	Apparent lignin g.	N in lignin mg.	Apparent lignin g.	N in lignin mg.
Lucerne	35.4	0.1503	6.9	0.1500	6.3	0.1510	7.9	0.1460	5.8
Rotted rice straw	5.2	0.1082	2.8	0.1194	2.2	0.1045	2.6	0.0887	2.3
Rotted rye straw	32.9	0.4680	13.8	0.4945	17.5	0.4403	14.9	0.4195	9.7
Rotted oat straw	18.6	0.3085	7.5	0.3010	7.8	0.2420	6.7	0.2565	5.2

in reducing the protein disturbance, but no other expedient either in the nature of a pretreatment or a modification of conditions achieves as much. Until an alternative is found, this treatment will be given, inasmuch as it minimises the effect of both protein and pentose. On theoretical grounds it should then be immaterial whether the time of contact with 72 % acid is 2 hours or 16 hours, but since with certain materials high in nitrogen the shorter periods caused a higher yield of apparent lignin and a higher content of nitrogen in lignin, the 16-hour procedure is preferable. In any case the nitrogen content of the lignin should be determined and recorded but no attempt made to apply any correction by calculating as protein and subtracting from the apparent lignin yield. Sufficient evidence has been given to indicate that this procedure may introduce errors larger than those which it is designed to correct.

SUMMARY.

1. Proteins alone give no precipitate on standing with 72 % H_2SO_4 but when added to plant materials increase the apparent lignin content. The lignin residue then obtained contains nitrogen.

2. The magnitude of the disturbance produced is quite different if the material is previously subjected to a hydrolytic pretreatment, thus indicating some interaction between the hydrolysable constituents and protein, which enhances the disturbing effect of both.

3. If xylose and protein are treated together with 72 % acid, insoluble precipitates are formed when the protein present is small in amount. Increasing quantities of protein give diminishing yields of precipitate, till none is formed. This sparing effect of larger quantities of protein has also been observed with plant materials.

4. By comparing the effects of additions of protein to untreated straws and to an equivalent amount of hydrolysed straw, it is possible to distinguish between the protein disturbance alone, that produced by pentose and that produced by the interaction of the two. Small additions of protein cause a proportionately greater disturbance than do larger amounts.

5. The protein disturbance is probably due to the linkage of protein fission products with lignin. Direct linkage between protein and lignin is unlikely because the ratio of increment produced to nitrogen present is very variable. To apply a correction by calculating the nitrogen in the lignin as protein and subtracting is useless and likely to introduce in some cases an error greater than that caused by the presence of nitrogenous material.

6. The magnitude of the disturbance cannot be reduced by decreasing the time of contact with acid from 16 to 2 hours or by following the Ritter-Seborg-Mitchell procedure.

7. Acid pretreatment results in a lowering of the error in most cases and though only partially successful has been provisionally adopted.

REFERENCES.

- Hobson and Page (1932). *J. Agric. Sci.* **22**, 497.
Paloheimo (1925). *Biochem. Z.* **165**, 463.
Phillips (1932). *J. Assoc. Off. Agric. Chem.* **15**, 124.
Ritter, Seborg and Mitchell (1932). *Ind. Eng. Chem., Anal. Edit.* **4**, 202.
Waksman and Iyer (1932). *J. Washington Acad. Sci.* **22**, 41.
— and Stevens (1930). *Ind. Eng. Chem., Anal. Edit.* **2**, 167.

XXXVIII. THE DETERMINATION OF AMINO-ACIDS IN WHEAT FLOUR.

By LESLIE WILLIAM SAMUEL.

From the Rothamsted Experimental Station, Harpenden, Herts.

(Received December 6th, 1933.)

THE work here reported was undertaken as a preliminary to the investigation of the changes in the amino-acid content of flour dough during fermentation. In order to study these changes it is necessary to stop the enzyme action at different times before extracting and estimating the amino-acid content, unless the extract can be prepared in a time negligibly short in comparison with the time that fermentation has proceeded, and provided that there is no production of amino-acid in the aqueous extract. A disadvantage of the freezing method employed by Brownlee and Bailey [1930] to inhibit enzyme action is that the enzymes are not destroyed, and may become active during the extraction if this is carried out at normal temperatures, so that observed results may not necessarily be due to fermentation in the dough.

Denham and Scott Blair [1927] found that the variations in amino-acid content of flour-water suspensions for the first few hours after mixing, which were described by Swanson and Tague [1916; 1917], also occurred in the clear extract of the flour and concluded that the proteolytic activity of a flour could be measured by the increase in amino-acid content of the extract. The method of estimation used by Denham and Scott Blair, however, is open to criticism, as shown later. Swanson and Tague [1916] found that the amino-acid content of a flour extract increased on standing, but as their estimations were made weekly they are not comparable with those of Denham and Scott Blair [1927]. Herd [1931] reported that "the change in amino-nitrogen content of the flour extracts is negligible in all cases over a period of 118 hours," but the change in titre reported by Denham and Scott Blair [1927] would not cause a significant change in the titres reported by Herd [1931], and differences in technique render the two sets of results not strictly comparable.

In view of these apparently conflicting statements it seemed advisable to investigate the change in amino-acid content of a flour extract on standing, particularly for the period immediately after the separation of the flour.

Methods based on the formaldehyde titration of Sørensen [1907, 1] and the alkalimetric titration of Foreman [1920] have been used in flour work, and the Sørensen method has always been found the most suitable, though in the data published by Cairns and Bailey [1928] on a comparison of the two methods, the Foreman titration was carried out in 50 % alcohol, and its rejection was therefore not entirely justified since in 50 % alcohol the polypeptides would titrate but only a part of the amino-acids [see Willstätter and Waldschmidt-Leitz, 1921].

EXPERIMENTAL.

Although the procedures described by Swanson and Tague [1916] (for the Sørensen titration), by Foreman [1920] and by Harris [1923] (for a modified Foreman titration) all gave results accurate to within a few per cent. of the theoretical figures with pure amino-acids, these methods were

found to be unsuitable for flour extracts, the Foreman and Harris methods because they involve the dilution of the extract with nine volumes of 95 % alcohol (and the amino-acid content of the extract is originally very small) and the Swanson and Tague method because the slight opalescence of the flour extract renders the final end-point with phenolphthalein or thymolphthalein somewhat indefinite. The technique recommended by Brown [1923] for the formaldehyde titration of bacteriological media was therefore tried and was found very satisfactory.

To 10 ml. of flour extract was added 0.2 ml. of phenol red solution and the solution titrated to p_H 8.0 with $N/14$ NaOH, using a micro-burette calibrated to 0.01 ml. The end-point was obtained by matching with a buffer solution of p_H 8.0 in a comparator block. This titration (stage 1) represents the titratable acidity of the extract. To the sample were then added 8 ml. of commercial 40 % formaldehyde, 0.18 ml. of indicator solution, and the mixture was again titrated to p_H 8.0. (0.18 ml. of indicator was added because the blank titration on the formaldehyde is about 1 ml.). This second stage titration represents the alkali required both to neutralise the amino-acid in solution and to bring the formaldehyde to p_H 8.0. In each case suitable screens of extract and of diluted extract were used in the buffer side of the comparator block. This second stage titration was corrected by subtracting from the titre a blank titration to p_H 8.0 of 8 ml. of formaldehyde diluted with 10 ml. of distilled water instead of bringing the formaldehyde to p_H 8.0 before adding to the flour extract. No change in the blank titration was ever observed during the few hours occupied by the experiments to be described.

The end-points obtained by this method were very sharp and distinct and were sensitive to less than 0.01 ml. of $N/14$ NaOH. An endeavour to increase the sensitivity of the method by using $N/100$ alkali was not very successful owing to lack of distinctness of the end-point. Other indicators offered no advantage.

It is recognised that, under the conditions of this work, titrating the test solution to a colour match with the standard buffer solution does not necessarily mean that the p_H of the test solution and the buffer solution are the same [see Halton and Fisher, 1928], but the conditions are at least reproducible. Moreover, the electrometric determination of the p_H of a solution containing 16 % of formaldehyde would introduce difficulties in the error of the junction potential with the formaldehyde solution.

Table I. *Amino-nitrogen in flour extract at various times.*

Titration	Time (mins.)	Stage 1, ml. $N/14$ alkali per 2 g. flour	Stage 2, mg. amino-N per 2 g. flour
Flour (a)			
1	0	0.21	0.30
2	10	0.21	0.30
3	20	0.21	0.30
4	60	0.22	0.30
5	90	0.22	0.29
6	120	0.21	0.30
7	165	0.21	0.31
8	225	0.21	0.30
9	345	0.22	0.30
10	495	0.21	0.31
Flour (b)			
1	0	0.24	0.30
2	10	0.24	0.30
3	25	0.23	0.31
4	40	0.22	0.30
5	75	0.23	0.30
6	120	0.22	0.30
Flour (c)			
1	0	0.31	0.41
2	20	0.32	0.42
3	40	0.32	0.41
4	70	0.32	0.41
5	120	0.33	0.41
6	180	0.32	0.41

For the preparation of the extract the method described by Denham and Scott Blair was used. Flour, equivalent to 40 g. dry flour, was extracted by shaking for five minutes with 200 ml. of distilled water to which toluene had been added, the mixture centrifuged and the slightly opalescent extract decanted through a filter. The filtrate was titrated immediately and after various intervals of time. Representative results, given in Table I, for three different flours, (a) from a typical mill mixture, (b) from English wheat, (c) a 30 % low grade flour, show that there was no change in the amino-acid content of the extract on standing in the presence of toluene, nor was there any change in titratable acidity.

Further experiments in the absence of toluene gave the same results, thus confirming the finding of Denham and Scott Blair that for short period experiments toluene did not affect the results. Similar results were obtained when the accuracy of the method was increased five-fold by titrating 50 ml. of solution instead of 10 ml., so that the failure to observe an increase in amino-acid content with time was not due to the insensitivity of the method. Experiments were conducted in which the extract was maintained at 25° and 37° respectively, but no increase in amino-acid titration was obtained.

That the flours used exhibited the phenomenon of increase in amino-acid content with increasing time of extraction is shown in Table II. This table also shows the increase in titratable acidity (stage 1) with increasing time of extraction. This increase is due, at least in part, to an increase in the buffer value of the extract, caused by the production of inorganic phosphate from phytin by the action of phytase [Bailey and Peterson, 1921], but may also be due to the production of organic acids by fermentation processes. The low-grade flour (c) shows a much greater increase in amino-nitrogen than flours (a) or (b).

Table II. *Increase in amino-nitrogen with time of extraction.*

Time of extraction	Stage 1, ml. $N/14$ alkali per 2 g. flour	Stage 2, mg. amino-N per 2 g. flour
Flour (a)		
5	0.19	0.30
20	0.27	0.32
35	0.32	0.35
50	0.33	0.35
Flour (b)		
5	0.24	0.30
15	0.28	0.33
30	0.33	0.33
55	0.40	0.37
75	0.44	0.38
105	0.49	0.39
Flour (c)		
5	0.31	0.42
30	0.56	0.48
60	0.73	0.54
120	0.85	0.55

Since the results shown in Table I are directly opposed to the findings of Denham and Scott Blair, the work was repeated, using the method of estimation described by these authors (an acid titration of one portion to p_H 5.5 with methyl red and an alkali titration of a second portion to "a full rose colour" with phenolphthalein¹ in the presence of formaldehyde) both in the presence and absence of toluene. With the mill mixture and English flours no increase in amino-acid content was obtained either with or without toluene, but the extract of the low-grade flour showed an increase in the titration with time whether

¹ For this end-point a buffer solution of p_H 9.0 was used in the work here described.

Table III. *Amino-nitrogen content of extract of low-grade flour (no toluene).*

Time mins.	Denham and Scott Blair method			Brown method	
	ml. acid N/14	ml. alkali N/14	Total amino-N mg. per 2 g. flour	Stage 1	Stage 2, mg. amino-N per 2 g. flour
0	0.11	0.77	0.88	0.32	0.41
20	0.11	0.85	0.96	0.31	0.41
40	0.11	0.88	0.99	0.31	0.40
60	0.12	0.90	1.02	0.31	0.41
120	0.14	0.91	1.05	0.31	0.41

toluene were present or not. Some typical figures are given in Table III which show an increase in the amino-nitrogen content of the extract as estimated by the method of Denham and Scott Blair and no increase by the technique of Brown. Table IV shows the absence of any change in amino-nitrogen content of the "mill mixture" and the English flour and the increase in the case of the low-grade flour by the Denham and Scott Blair method irrespective of the presence or absence of toluene.

Table IV. *Amino-nitrogen content of flour extracts with and without toluene.*

Time mins.	Denham and Scott Blair method					
	Without toluene			With toluene		
	ml. acid N/14	ml. alkali N/14	Total amino-N mg. per 2 g. flour	ml. acid N/14	ml. alkali N/14	Total amino-N mg. per 2 g. flour
English flour						
0	0.06	0.63	0.69	0.06	0.63	0.69
30	0.06	0.62	0.68	0.06	0.63	0.69
60	0.06	0.64	0.70	0.06	0.63	0.69
120	0.06	0.63	0.69	0.06	0.63	0.69
240	0.06	0.63	0.69	0.06	0.63	0.69
Mill mixture						
0	0.07	0.60	0.67	0.07	0.61	0.68
30	0.07	0.60	0.67	0.07	0.60	0.67
60	0.07	0.61	0.68	0.07	0.61	0.68
120	0.07	0.61	0.68	0.07	0.61	0.68
Low grade flour						
0	0.10	0.79	0.89	0.11	0.80	0.91
30	0.11	0.88	0.99	0.11	0.89	1.00
60	0.11	0.92	1.03	0.12	0.92	1.04
120	0.12	0.94	1.06	0.13	0.94	1.07
180	0.13	0.96	1.09	0.13	0.95	1.08

The slight discrepancies between the amino-nitrogen content of the low grade extract with and without toluene are due to the technique of this experiment which was to prepare several hundred ml. of extract, divide this into two portions and add toluene to one portion. Each portion was then titrated in turn, so that before the initial titration the "toluene" extract had stood a slightly longer period than had the "no toluene" extract, and this is reflected in the slightly higher amino-nitrogen content of the "toluene" extract.

Table III also illustrates the great difference in the absolute magnitude of the amino-acid content as determined by the two methods; that obtained by the Denham and Scott Blair method (the sum of the two titrations) being some two to three times as great as that obtained by titrating to the same p_H before and after the addition of the formalin solution. This discrepancy is due to the titratable acidity and buffer capacity of the flour extract which are included as amino-acid by the Denham and Scott Blair method.

Though the main object of this investigation had been accomplished in showing that no measurable proteolysis occurred in the flour extracts for the few hours immediately subsequent to the separation of the flour, it was thought to be of interest to try and explain the anomalous result obtained with the low-grade flour. Extracts of this flour, prepared as described above, were analysed in the following manner after various periods of standing. 10 ml. of extract were titrated with $N/14$ HCl to p_H 5.5 as in the Denham and Scott Blair method. To another 10 ml. was added 0.2 ml. of thymol blue indicator and the solution titrated to match successively the colours in buffers of p_H 8.0, 8.5, 8.7 and 9.0; formaldehyde and further indicator solution were then added and the mixture again titrated to these end-points. Table V shows the results obtained in one such experiment when toluene water was used for the extraction. The amino-acid by the method of Denham and Scott Blair is the sum of the titration to p_H 5.5, the titratable acidity and the amino-acid by the method of Brown.

From Table V it is seen that there is no change in the buffer value of the solution as shown by the acid titration to p_H 5.5 and the alkali titrations to

Table V. *Determination of amino-acid at various times in the aqueous extract of a low-grade flour by the methods of Brown and of Denham and Scott Blair.*

Time in mins.		{from first titration from mixing		0	20	40	60	120
				20	40	60	80	140
Acid titration to p_H 5.5; ml. $N/14$ HCl				0.10	0.10	0.10	0.10	0.10
p_H 8.0	{ Titration before adding formaldehyde; ml. $N/14$ NaOH			0.31	0.31	0.31	0.31	0.31
	{ Amino-nitrogen mg. per 2 g. flour	Brown	Denham and Scott Blair	0.42 0.83	0.41 0.82	0.42 0.83	0.42 0.83	0.41 0.82
p_H 8.5	{ Titration before adding formaldehyde; ml. $N/14$ NaOH			0.39	0.38	0.39	0.39	0.38
	{ Amino-nitrogen mg. per 2 g. flour	Brown	Denham and Scott Blair	0.38 0.87	0.38 0.86	0.38 0.87	0.38 0.87	0.38 0.86
p_H 8.7	{ Titration before adding formaldehyde; ml. $N/14$ NaOH			0.41	0.40	0.41	0.42	0.41
	{ Amino-nitrogen mg. per 2 g. flour	Brown	Denham and Scott Blair	0.36 0.87	0.35 0.85	0.35 0.86	0.34 0.86	0.35 0.86
p_H 9.0	{ Titration before adding formaldehyde; ml. $N/14$ NaOH			0.48	0.48	0.48	0.48	0.48
	{ Amino-nitrogen mg. per 2 g. flour	Brown	Denham and Scott Blair	0.28 0.86	0.33 0.91	0.38 0.96	0.40 0.99	0.48 1.06

p_H 8.0, 8.5, 8.7 or 9.0 respectively. Also this latter titration indicates that there is no change in the titratable acidity of the extract on standing. The amino-acid by the method of Brown is less the higher the p_H to which the solution is titrated, in accordance with the theory of the titration. At p_H 8.0, 8.5 and 8.7 there is no increase in amino-acid as estimated by either method, but at p_H 9.0 there is an increase by both methods.

It was suspected that this increase at p_H 9.0 was due to the production by enzyme action of substances containing both carboxyl and amino-groups but which did not become titratable even under the action of formaldehyde until p_H 9.0 was reached. If so, such increase might be enhanced were the enzyme

allowed to act on the flour substrate instead of on extracted protein only. A suspension of 40 g. of "dry" flour in 200 ml. of distilled water was allowed to autolyse at 27°. From time to time samples were taken, centrifuged, filtered and the filtrate titrated to p_H 8.0, 8.5, 8.7 and 9.0 respectively as before. The results of one such experiment are shown graphically in Fig. 1, plotting amino-nitrogen against time.

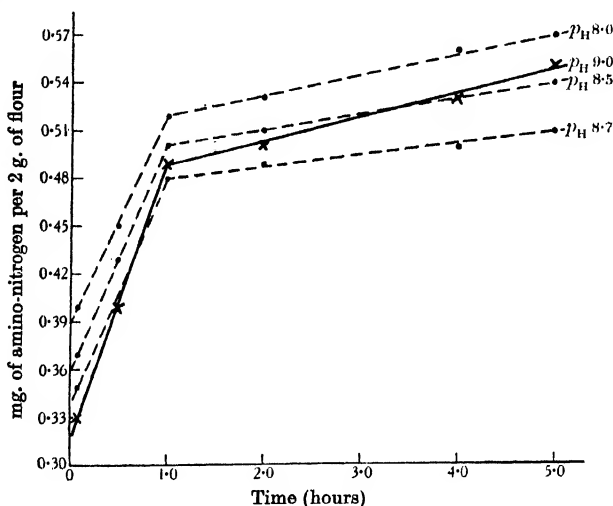


Fig. 1. Amino-nitrogen in flour-water digest at various times.

Initially the amino-acid content is lower the higher the p_H used, as is to be expected, and all values increase with time. This increase is due to further extraction¹ of the amino-acids from the flour and also to the production of amino-acids from the proteins. The rapid increase for about an hour is due almost entirely to extraction, the subsequent slow increase is due to proteolysis, and the slope of this second portion of the graph measures the proteolytic activity of the flour. The graphs at p_H 8.0, 8.5 and 8.7 are practically parallel, but the graph for p_H 9.0 crosses the other graphs, indicating the more rapid production of substances titratable at p_H 9.0.

The addition of yeast to a flour-water suspension did not enhance this effect, because the amino-acid produced was used by the yeast in its metabolism, and this rendered difficult the interpretation of the results from the standpoint of the present work. The indications were, however, that at each estimation after the first a greater titration was obtained at p_H 9.0 than at p_H 8.0 after the addition of formaldehyde. There was also evidence that the titration at p_H 8.7 was increased in the presence of yeast.

Table VI shows the results obtained from a takadiastase digest of the gluten washed from this flour. The amino-nitrogen estimated at p_H 9.0 increases more

¹ It may be noted that extraction was complete in one hour, so that the method of estimating amino-acids in flour given in the Methods of Analysis of the Association of Official Agricultural Chemists, p. 168 (3rd edition, 1930), which requires extraction for a three-hour period, measures not only the amino-acid originally present, but also that produced by some two hours' proteolysis, unless the extraction be carried out at a low temperature.

Table VI. *Amino-nitrogen (mg. per 10 ml. of solution) formed in a gluten-takadiastase digest.*

Time hours	Titra- tion	p_H 8.0		p_H 8.5		p_H 8.7		p_H 9.0	
		0.06	0.00	0.06	0.00	<0.07	0.00	<0.08	0.00
0	1								
	2								
1	1	0.10		0.12		0.13		0.14	
	2		0.05		0.03		0.02		0.02
2	1	0.12		0.14		0.15		0.16	
	2		0.06		0.04		0.06		0.07
3	1	0.14		0.16		0.17		0.18	
	2		0.09		0.07		0.09		0.10
4	1	0.16		0.18		0.19		0.21	
	2		0.12		0.10		0.12		0.13
5	1	0.18		0.20		0.22		0.23	
	2		0.14		0.12		0.14		0.18

Titration 1: initial alkali titration.

Titration 2: alkali titration in presence of formaldehyde less the formaldehyde blank, i.e. amino-nitrogen.

rapidly than that at p_H 8.0. In all cases the amino-nitrogen at p_H 8.5 is less than that at p_H 8.0 for the corresponding time in accordance with theory, but it is clear that the substances affecting the estimation at p_H 9.0 are also exercising an influence at p_H 8.7, but to a lesser extent. The action of takadiastase on the gluten is thus comparable with the changes occurring in the flour extract and in the flour suspension.

No such abnormal increase at p_H 9.0 was obtained in pepsin digests of gluten, caseinogen or egg-albumin.

An acid hydrolysis of the moist gluten from this low-grade flour was followed by estimation of the amino-acid from time to time by titration to p_H 8.0, 8.5, 8.7 and 9.0 respectively as before. The results shown in Table VII indicate that hydrolysis was normal, and that the amino-acid estimated at p_H 9.0 is always less than that at p_H 8.0.

Table VII. *Amino-acid formed in acid hydrolysis of flour-gluten.*

Time hours	mg. of amino-nitrogen at			
	p_H 8.0	p_H 8.5	p_H 8.7	p_H 9.0
0	0.00	0.00	0.00	0.00
0.5	0.65	0.62	0.60	0.52
1.0	0.84	0.81	0.80	0.76
2.0	0.93	0.88	0.88	0.82
3.0	1.02	0.98	0.98	0.91
6.0	1.06	1.03	1.03	0.98
9.0	1.09	1.05	1.05	0.98

To determine, if possible, the class of substance responsible for the abnormal increase, various materials probably occurring in the flour-water suspension were titrated to p_H 8.0, 8.5, 8.7 and 9.0 respectively before and after the addition of formalin. Neither protein, peptone nor amino-acid solutions showed any increased titration at p_H 9.0. Indeed all showed the expected decrease in titration with increase in p_H value. These results are shown in Table VIII. All possible mixtures of these solutions were titrated similarly, and the values obtained were strictly additive and gave no evidence of an increased titration

at p_H 9.0 but showed the same reduction in titration as is illustrated in Table VIII. In each case the requisite amount of solution was taken, diluted to 10 ml. and then titrated.

Table VIII. *Amino-acid estimation at p_H 8.0, 8.5, 8.7 and 9.0 respectively.*

Titration ml. $N/14$ NaOH		Phenol red		Thymol blue			
		p_H 7.0	p_H 8.0	p_H 8.0	p_H 8.5	p_H 8.7	p_H 9.0
Egg-albumin	1	0.03	0.08	0.08	0.09	0.10	0.11
	2	0.21	0.25	0.25	0.24	0.23	0.20
Peptone	1	0.06	0.11	0.11	0.16	0.18	0.20
	2	0.18	0.18	0.18	0.15	0.14	0.09
Alanine (theory 0.91 ml.)	1	0.01	0.02	0.02	0.06	0.09	0.12
	2	0.74	0.89	0.89	0.87	0.86	0.81
Glutamic acid hydrochloride (theory 0.91 ml.)	1	1.82	1.83	1.83	1.89	1.92	2.01
	2	0.57	0.88	0.88	0.83	0.81	0.76

Titration 1: solution without formaldehyde.

Titration 2: difference between titrations before and after the addition of formaldehyde corrected for the blank titration, i.e. titration due to amino-acid.

Owing to the large titration to neutralise the solution of glutamic acid hydrochloride, extra indicator was added to obtain the same indicator concentration as in the buffer solution. Also the formaldehyde blank was determined on 8 ml. of formalin diluted with 12 ml. of distilled water.

DISCUSSION.

The results obtained in this study of the estimation of amino-nitrogen in wheat flour show that extraction is complete in about one hour, and that no further formation of amino-acid occurs in the extract after separation from the flour. In the case of one flour, a low-grade, there was an apparent increase in amino-nitrogen in the extract when the estimation was carried out at p_H 9.0, but no increase at the lower p_H values used.

This increase is not due to more complete neutralisation of the amino-acids at the higher p_H , for the more recently published titration curves for individual amino-acids in the presence of formaldehyde show that neutralisation is complete at p_H 8.0¹ [Brown, 1923; Harris, 1929]. In Table VIII the low results at p_H 7.0 are due to incomplete neutralisation of the amino-acid in the presence of formaldehyde, and the low results at $p_H > 8.0$ are due to partial neutralisation of the amino-acids before the addition of formaldehyde. That the maximum titration at p_H 8.0 is slightly less than the theoretical titration value of 0.91 ml. is also due to this latter factor.

Moreover, the facts that in the case of the flour extract, the flour suspension and the takadiastase-gluten digest, lower titrations were recorded at p_H 8.5 than at p_H 8.0, and that there is not a progressive increase in titration with increase in p_H indicate that completion of neutralisation of the amino-acids is not the explanation of the phenomenon.

If it be accepted that the formaldehyde titration estimates carboxyl groups that become titratable because of the action of formaldehyde on the amino-groups, then the observed increase at p_H 9.0 is due to an increase in the number

¹ Since the above was written a recent paper by Van Slyke and Kirk [1933] has been received which shows titration curves of amino-acids compiled from various authors. The titration curves in the presence of formaldehyde show that neutralisation is not complete before p_H 9.0. However, these curves were "estimated from end-points at p_H 9 to 10, given by Sørensen" [1907, 2], and are said to be "only approximately exact" [Van Slyke and Kirk, 1933, p. 657], so that the experimental curves of Brown [1923] and of Harris [1929] are the more reliable.

of carboxyl groups (or amino-groups affected by formaldehyde) which, even in the presence of formaldehyde, do not titrate until p_H 9.0 is reached. That such increase is not due to the production of amino-acids in the usual sense of this term is shown by the titration values at p_H 8.0. Nor is this increase due to the production of proteoses or peptones, as shown by a pepsin digestion, though it occurs in a takadiastase digest of gluten. It is therefore due to substances formed in the enzymic hydrolysis of the proteoses or peptones formed from gluten. A combination of carboxyl and amino-groups in a compound of very low acid dissociation constant may be expected in a substance intermediate between the polypeptides and the amino-acids. Such a substance, under the action of formaldehyde, would not affect the amino-acid estimation at p_H 8.0 because of its very low acid dissociation constant but would become titratable at p_H 9.0. Then in the flour extract, if the proteins present are broken down, not to their constituent amino-acids but to some slightly larger grouping of a few amino-acids, this would be estimated at p_H 9.0 and not at p_H 8.0 and would explain the experimental results.

Although only one of the four flours examined to date has shown this phenomenon of increase in amino-nitrogen at p_H 9.0 and not at p_H 8.0, it seems from the work of Denham and Scott Blair and of Halton (unpublished) that it may be of more common occurrence than this would indicate.

In considering the question of protein changes in a flour dough it is necessary to take into account any such change as is indicated in this paper, for the physical properties of the dough will be affected by any protein degradation whether the decomposition be carried as far as the amino-acid stage or not.

Perhaps it should be emphasised that titration to p_H 9.0 before and after the addition of formaldehyde is not advocated as a method of estimating amino-acids, but that in conjunction with an estimation at p_H 8.0 it may yield some information not afforded by the amino-acid estimation alone.

SUMMARY.

1. The claim that proteolysis occurs in flour extracts has been studied for the few hours immediately subsequent to separation of the extract.
2. The technique of Brown for the estimation of amino-acids by the Sørensen method has been applied to flour extracts.
3. Proteolysis, as measured by the production of amino-acids, did not occur in the flour extracts studied.
4. For one flour of the four studied (a low-grade) the amino-acid determinations made on the extract at varying p_H values showed an increase with time if the solution were titrated to p_H 9.0 before and after the addition of formaldehyde, but no increase if the titration were carried to p_H 8.0 only.
5. It is suggested that this is due to the enzymic production of substances containing carboxyl and amino-groups and having a low acid dissociation constant, which do not titrate even in the presence of formaldehyde until p_H 9.0 is reached.

The author is indebted to the University of Western Australia for the award of a Hackett Research Studentship during the tenure of which this work was carried out, to Sir John Russell, Director of Rothamsted Experimental Station, for placing at his disposal the facilities of the Station, and to the Research Association of British Flour Millers for the flours used in this work. His thanks are due to Dr A. G. Norman, Dr P. Halton and Mr G. W. Scott Blair for their assistance and advice.

REFERENCES.

- Bailey and Peterson (1921). *J. Ind. Eng. Chem.* **13**, 916.
Brownlee and Bailey (1930). *Cereal Chem.* **7**, 487.
Brown (1923). *J. Bact.* **8**, 245.
Cairns and Bailey (1928). *Cereal Chem.* **5**, 79.
Denham and Scott Blair (1927). *Cereal Chem.* **4**, 58.
Foreman (1920). *Biochem. J.* **14**, 451.
Halton and Fisher (1928). *Cereal Chem.* **5**, 445.
Harris (1923). *Proc. Roy. Soc. Lond. B* **95**, 500.
—— (1929). *Proc. Roy. Soc. Lond. B* **104**, 412.
Herd (1931). *Cereal Chem.* **8**, 145.
Sørensen (1907, 1). *Biochem. Z.* **7**, 45.
—— (1907, 2). *Compt. Rend. Lab. Carlsberg*, **7**, 1.
Swanson and Tague (1916). *J. Amer. Chem. Soc.* **38**, 1098.
—— ——— (1917). *J. Amer. Chem. Soc.* **39**, 482.
Van Slyke and Kirk (1933). *J. Biol. Chem.* **102**, 651.
Willstätter and Waldschmidt-Leitz (1921). *Ber. deutsch. chem. Ges.* **54**, 2988.

THE DECOMPOSITION OF GREEN MANURES IN SOIL¹.

By J. A. DAJI.

(*The Fermentation Department, Rothamsted Experimental Station,
Harpenden, Herts.*)

(With Eleven Text-figures.)

THE decomposition of green manures in soil has been very extensively studied, but most of the work has been done mainly to measure the rate of formation of the end products of decomposition. The evolution of carbon dioxide has been used by many workers as a criterion for the decay of organic matter. The production of ammonia and the ultimate accumulation of nitrates have been utilised by others to measure the rate of decomposition. Neither of these measurements, however, supplies precise information concerning the processes that govern the liberation of plant nutrients in an available form.

When a green manure is turned under, the micro-organisms present in the soil break down the complex plant material into simpler substances, part of which they consume for building their body tissues, leaving the rest as by-products. The decomposition of a plant material is, therefore, controlled by the organisms that exist in the soil and the environmental conditions under which they function on the one hand, and by the type and variety of the chemical complexes, of which the plant is composed, on the other.

In the present paper the decomposition of green manures in soil under laboratory conditions has been followed by determining periodically the changes taking place in the various plant constituents, such as the carbohydrates and allied products (Series I) and the nitrogen transformations (Series II).

SERIES I.

Experimental.

Four substances—young tares, young mustard, sugar-beet tops and mature mustard—were used. All the materials were rapidly dried at 55–60° C. and finely powdered. The light sandy soil employed was ob-

¹ This paper is an abridged form of the thesis approved for the Degree of Doctor of Philosophy in the University of London.

16 *The Decomposition of Green Manures in Soil*

tained from the headland of one of the green manure experimental fields at the Woburn Experimental Station. It was air dried and passed through a 1 mm. sieve. Each material was added to the soil at the rate of 5 per cent. on the air-dry basis, and water was carefully mixed in to bring the water content of the whole to about 15 per cent. The mixture was put into shallow Petri dishes and incubated at air temperature in a room that was not subject to great fluctuations of temperature. The dishes were kept loosely covered with other Petri dishes of the same size to exclude dust and to reduce evaporation, while allowing free access of air. The loss of moisture was made up very frequently and the samples were aerated by stirring. Separate samples were incubated for the different stages of decomposition. Five sets of soil samples were laid out in this way, one for each of the four green manures and the fifth for the soil alone, which served as a control.

The composition of the green manures as analysed by the methods outlined below is given in Table I. The same determinations, with the exception of lignin and ether extract, were made on the decomposing mixtures.

(1) *Total organic matter.* This was estimated by the loss of weight on ignition.

(2) *Water-soluble organic matter.* An aliquot quantity of the filtrate, representing 1 gm. of the material, obtained by shaking with 100 parts of water for 1 hour, was evaporated to dryness and the loss of weight on ignition was taken as water-soluble organic matter. In the case of soil, 25 gm. were shaken with 125 c.c. water and 50 c.c. taken for evaporation. The ash obtained from this determination was taken as soluble mineral matter.

(3) *Ether extract.* 1 gm. of the substance was extracted with ether in a Soxhlet extractor; the extract was evaporated and the residue weighed.

(4) *Total furfuraldehyde.* This was determined by the usual method of distillation with 12 per cent. HCl and precipitation with phloroglucinol. 20 gm. of the soil were used for the determination.

(5) *Cellulose.* Cellulose was determined by the method described elsewhere (2).

(6) *Lignin.* Lignin was determined by the Schwalbe method as described by Norman (4).

(7) *Nitrogen.* By the Kjeldahl method.

(8) *Soil reaction.* pH values were determined by the quinhydrone method.

Table I. *Chemical composition of the green manures and of soil, expressed on 100 gm. dry matter.*

	Young tares	Young mustard	Sugar-beet tops	Mature mustard	Soil
Total organic matter	66.60	73.62	81.57	90.81	2.920
Water-soluble organic matter	11.65	22.40	40.20	19.40	0.025
Water-soluble mineral matter	5.35	8.00	7.00	3.70	0.016
Ether extract	2.24	2.43	1.90	1.14	—
Total furfuraldehyde	5.45	6.40	6.65	12.10	0.089
Cellulose	9.71	9.01	7.39	23.32	0.060
Lignin	12.57	13.76	7.38	20.90	—
Nitrogen	3.70	3.65	2.74	1.52	0.114
Potash (K_2O)	2.90	3.85	5.98	—	—

Results.

The decomposition undergone by the various constituents of the green manures is shown in Figs. 1-4. The figures represent the percentage loss of each constituent. They were obtained by subtracting the amount of each of the constituents present in the control soil at the different periods of incubation from those present in the manured soils calculated back to the original material. The percentage loss was worked out therefrom.

Discussion.

There is a very rapid fall of soluble organic substances during the first 4-8 days in the case of all the four plant materials. The rate then falls off and there is very little decomposition after 25 days. Mature mustard contains almost as much soluble organic material as young mustard, yet it loses appreciably more, especially during the first week. The rapidity of decomposition of hemicelluloses as judged by the loss of total furfuraldehyde is about the same in the case of young tares, young mustard and sugar-beet tops. Mature mustard, however, decomposes more slowly though it contains a greater abundance of this group of substances. This is because it contains less soluble nitrogenous compounds than the other three (Series II).

There is no appreciable loss of cellulose during the first 4 days, whereas a considerable amount of the soluble organic matter and hemicelluloses decomposed during that time. The easy and rapid availability of these two latter classes of compounds to the micro-organisms during the opening stages of decomposition is thus evident. A great loss of cellulose takes place during the next 4 days in the case of young tares, young mustard and sugar-beet tops. The rate of disappearance of cellulose in mature mustard is slower on the whole. The comparatively slow

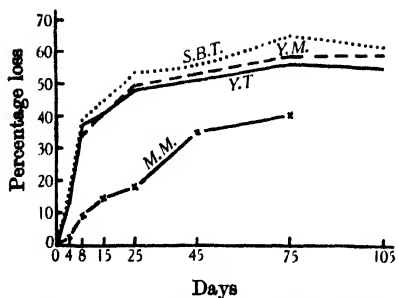


Fig. 1. Decomposition of total organic matter.

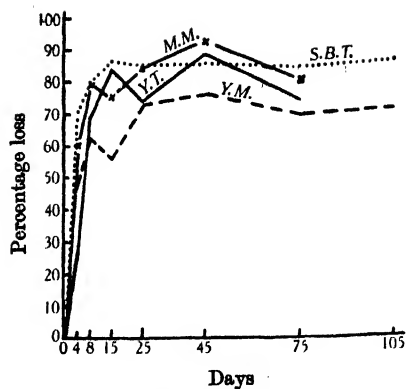


Fig. 2. Decomposition of soluble organic matter.

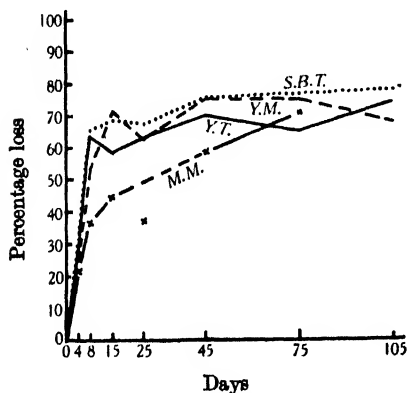


Fig. 3. Decomposition of total furfuraldehyde.

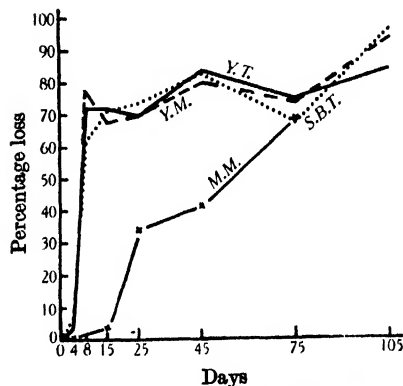


Fig. 4. Decomposition of cellulose.

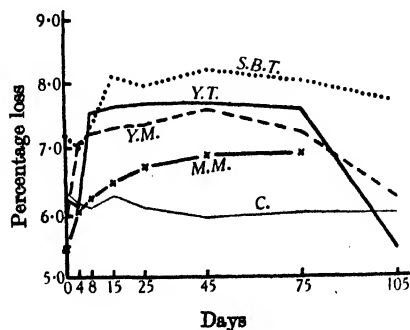


Fig. 5. Soil reaction.

S.B.T. Sugar-beet tops. *Y.M.* Young mustard. *Y.T.* Young tares.
M.M. Mature mustard. *C.* Control.

decomposition of cellulose in mature mustard is principally due to the high content of lignin which, by virtue of its encrustation on the cell walls, acts as a barrier to the utilisation of cellulose by micro-organisms. But, in view of the fact that the proportion of lignin to cellulose is much higher in the case of young tares, young mustard and sugar-beet tops than in mature mustard, it seems that the rapid availability of cellulose in the young plant materials is only apparent. It is because of the low cellulose content of these three plant materials as compared with their available nitrogen contents that almost the whole of that cellulose is required and used up by the micro-organisms. In other words the amount of available nitrogen in these three plant materials is more than sufficient to balance the total available carbohydrate, viz. the sugars, the hemicelluloses and cellulose. The reverse is true in the case of mature mustard, so that, after the first rapid removal of the soluble carbohydrates together with the soluble nitrogenous compounds, the other carbohydrate compounds decompose slowly as the rest of the nitrogenous substances become available. This is more clearly shown in the data presented in Table II. The sum of the soluble carbohydrates (soluble organic matter — total soluble N \times 6.25), hemicelluloses (total furfural \times 2), and cellulose is taken as the total available energy material and the sum of the total water-soluble nitrogen plus the nitrogen hydrolysed by H_2O_2 (Series II) is taken as the total available nitrogen. It has been shown that for each part of nitrogen assimilated, fifty to sixty parts of cellulose⁽¹²⁾ and twenty to forty parts of hemicelluloses⁽¹³⁾ are decomposed. Taking an average of these figures, the energy material that would be required for the total amount of available nitrogen present in each plant material is calculated and shown in the last column of the table.

Table II.

	Total energy material present	Total available nitrogen present	Energy material equivalent to available nitrogen (calculated)
Young tares	26.1	2.47	105
Young mustard	37.6	2.13	90
Sugar-beet tops	54.5	1.74	73
Mature mustard	63.7	0.81	34

Young tares, young mustard and sugar-beet tops contain relatively little carbohydrate in proportion to the available nitrogenous compounds present. Hence a very rapid and immediate decomposition takes place.

The total organic matter disappeared less slowly in the case of all

the four plant materials than the hemicelluloses, cellulose and soluble organic matter. This is primarily due to the accumulation of the more resistant constituents, chiefly lignin, which does not decompose as readily as the other plant constituents. Lignin was not determined in the decomposing materials, mainly because there is no suitable method for estimating it in a mixture of soil and plant material. The formation of new organic matter in the tissues of the fungal mycelium and bacteria is also a cause of the less rapid decomposition of total organic matter.

These results are in keeping with the findings of Rege (7), Norman (5), Waksman and his associates (13, 14, 15) and others cited by Tenney and Waksman (11). Rege first observed that in mature plant materials pentosans form the principal food material for the micro-organisms. This was later confirmed by Norman, who emphasised the importance of cellulose along with the pentosans as the chief source of energy material for the organisms. He further supported the view of Rege that lignin acts as an inhibitory agent. The work of Waksman and his colleagues showed that the decomposition depends on the nature and age of the plant material. The younger the plant the more rapidly does it decompose. The decomposition is influenced by the type of compounds present in the plant material, some like sugars, hemicelluloses and cellulose being more easily available than the others, *e.g.* lignin, resin and waxes. In confirming the previous work of Hutchinson and Richards (3) they further showed that there is a definite proportion between the amounts of available carbohydrate and available nitrogen for the requirements of the activities of the micro-organisms. Hence plants having a balanced proportion of these two classes of compounds decomposed more rapidly than others.

The nitrogen figures are more fully discussed in the second series of experiments.

The effect of green manures on the soil reaction is well marked (Fig. 5). After a small increase of acidity during the initial stages of decomposition the soil tends to become alkaline and remains so for a major part of the period of incubation. The increase in acidity and then in alkalinity seems to correspond with the production of organic acids from sugars and starches followed by, or simultaneously with, the production of ammonia. The fall in *pH* values during the later stages of decomposition corresponds to the formation of nitrates (Series II). Soil mixed with sugar-beet tops, however, remains alkaline even after nitrification has set in. This is due to the large amount of potash in the sugar-beet tops, which tends to keep the soil alkaline. Other differences in

the soil reaction due to the various green manures can also be similarly explained. The reaction of the soil is controlled by two factors: (1) the products of decomposition, viz. the organic acids, ammonia and nitrates, and (2) the liberation of the ash constituents of the green manure. The preponderance of one over the other decides the condition of the soil at any given time. The work of White⁽¹⁶⁾, Stephenson^(9, 10) and Smith and Humfield⁽⁸⁾ goes to show that the soil becomes alkaline during the early stages of decomposition but becomes acidic later on.

SERIES II.

The second series of experiments was designed to determine the nitrogen changes that take place during the decomposition of the green manures under study. Besides measuring the accumulation of ammonia and nitrates an attempt was made to observe the changes taking place in the potentially available class of compounds other than those soluble in water.

Mixtures of soil plus green manures were laid out in Petri dishes in the same way as in Series I, and samples were taken for analysis at the same intervals but for 65 days only.

The following determinations were carried out:

(1) *Total nitrogen*. Total nitrogen was determined by the salicylic-thiosulphate method to include nitrate nitrogen⁽¹⁾.

(2) *Water-soluble ammonia nitrogen*. A suitable quantity of the soil was shaken with five times its weight of water for 1 hour; an aliquot volume of the clear filtrate representing 20 gm. of the soil was distilled with magnesia and ammonia collected in standard acid.

(3) *Nitrate nitrogen*. Nitrate nitrogen was determined from the same filtrate as the ammonia, the contents of the flask being made up with water and 2-3 gm. of Devarda alloy added.

(4) *Total water-soluble nitrogen*. On another aliquot from the soil extract total soluble nitrogen was determined according to Ranker⁽⁶⁾. Total soluble nitrogen minus ammonia plus nitrate nitrogen is taken as water-soluble protein nitrogen.

(5) *Hydrogen peroxide nitrogen*. The action of dilute H_2O_2 was used to determine the potentially available class of nitrogenous compounds. The residue after water extraction was treated with 60 c.c. of a 3 per cent. solution of hydrogen peroxide. After the initial frothing had ceased, it was boiled for 15 min. The extract was filtered through a Buchner funnel, the residue washed and the filtrate made up to a definite volume. An aliquot portion of the filtrate was distilled with magnesia

and another with caustic soda. Each time ammonia was collected in separate quantities of standard acid. The residual soil was then further treated with another 60 c.c. of 3 per cent. hydrogen peroxide and ammonia determined as in the previous case. A third extraction was then made on the residual material and ammonia determined again as before. Thus each sample gave two forms of nitrogen three times in succession.

Assuming that the oxidising power of 3 per cent. hydrogen peroxide, under standard conditions of time and volume, measures the availability of the less easily available class of compounds, this group is sub-divided into two classes: (1) that which would be more easily available; this is measured by the distillation of the extract with magnesia and called " H_2O_2 nitrogen I," and (2) the rest of the nitrogen, which is measured by the difference between the nitrogen evolved by the caustic soda distillation and that by the magnesia distillation. This form of nitrogen is referred to as " H_2O_2 nitrogen II." The nitrogen evolved by the caustic soda distillation is referred to as the "Total H_2O_2 nitrogen."

The green manures were subjected to this system of analysis, the results of which are given in Table III.

Table III. *Different forms of nitrogen in the green manures, expressed on 100 gm. dry matter.*

		Young tares	Young mustard	Sugar- beet tops	Mature mustard
1.	Total nitrogen	3.70	3.65	2.74	1.52
2.	Water-soluble nitrogen				
	Ammonia nitrogen	0.096	0.249	0.089	0.049
	Nitrate nitrogen	0.096	0.121	0.160	0.034
	Total soluble nitrogen	1.18	1.43	1.27	0.60
	Percentage of total nitrogen	31.90	39.20	46.30	39.50
3.	H_2O_2 nitrogen I				
	1st extraction	0.460	0.114	0.124	0.029
	2nd extraction	0.226	0.179	0.049	0.032
	3rd extraction	0.051	0.111	0.041	0.019
4.	H_2O_2 nitrogen II				
	1st extraction	0.383	0.102	0.162	0.056
	2nd extraction	0.169	0.133	0.041	0.047
	3rd extraction	0.002	0.064	0.052	0.031
5.	Total available nitrogen (2 + 3 + 4)	2.47	2.13	1.74	0.81

It is interesting to note that mature mustard contains relatively as much nitrogen in a soluble form as young mustard. It is the nitrogen soluble in water which, according to Whiting and Richmond (17), is responsible for the early nitrification of plant materials even though they may contain less total nitrogen. Young tares contain considerably more nitrogen oxidised by H_2O_2 both absolutely as well as relatively to

the total nitrogen, than the other three plant materials. Looking to the nitrogen oxidised at each of the extractions it will be noticed that young tares and sugar-beet tops liberate a major portion of the nitrogen at the first extraction both as H_2O_2 nitrogen I as well as H_2O_2 nitrogen II. In the case of young as well as mature mustard, however, it is a more gradual liberation.

RESULTS AND DISCUSSION.

The results of the decomposition studies of the four green manures are presented in Figs. 6–11. As the nitrogen liberated by hydrogen peroxide at the three different extractions did not show any noticeable differences, the total of the three extractions is given together. All the results have been calculated on the original dry matter and represent nitrogen present in the soil mixtures at different periods.

The loss of total nitrogen, especially in the case of young tares, young mustard and sugar-beet tops is considerable. A similar loss of nitrogen was noticed in the first series of experiments. It was then not clear whether that loss was actual or only apparent as the nitrogen determinations did not include nitrates. In the present series of experiments, where the total nitrogen was determined so as to include nitrates, the loss becomes actual. It has been shown previously (Table II) that these three plant materials have a very high proportion of total available nitrogen to total energy material. In other words they have a narrow nitrogen-carbon ratio. This accounts for the loss of nitrogen noticed in these experiments.

Similar losses of nitrogen have been observed by other workers. Tenney and Waksman (11) noticed a decrease of total protein during the decomposition of alfalfa which contained 2.58 per cent. total nitrogen. But the more striking experiments are those of Zolcinski and Musierowicz (18, 19) who, while studying the decomposition of lucerne and red clover, noticed large losses of total nitrogen. These are materials both very rich in nitrogen. The present experiments are, therefore, in accord with the results obtained by them.

The opening stage of the decomposition is characterised by a rapid loss of soluble nitrogen during the first 4 days (Fig. 7). During the same time there is an increase in the amount of total H_2O_2 nitrogen (Fig. 10). It seems therefore that the soluble nitrogen is being converted into more complex nitrogenous compounds, very probably the synthesised protein matter of the fungi that are growing very vigorously at that time. A part of the increase in total H_2O_2 nitrogen may also be due to the

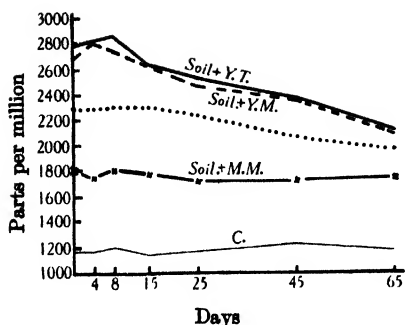


Fig. 6. Changes in total nitrogen.

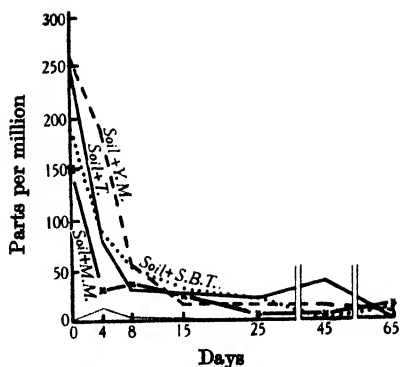


Fig. 7. Changes in water-soluble protein nitrogen.

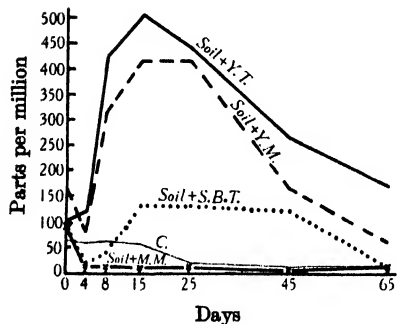


Fig. 8. Changes in water-soluble ammoniacal nitrogen.

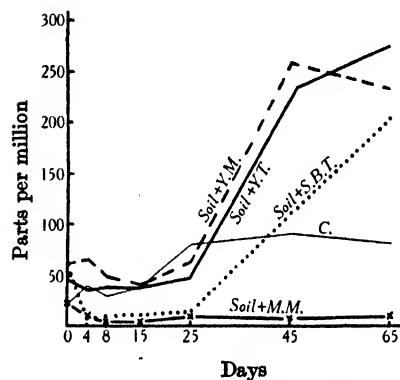


Fig. 9. Changes in nitrate nitrogen.

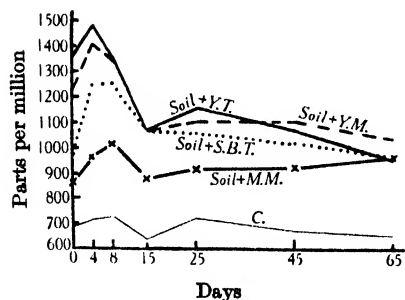


Fig. 10. Changes in total hydrogen peroxide nitrogen.

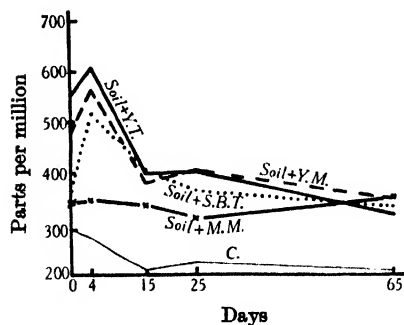


Fig. 11. Changes in hydrogen peroxide nitrogen II.

Soil + Y.T. Soil + young tares.
Soil + S.B.T. Soil + sugar-beet tops.

Soil + Y.M. Soil + young mustard.
Soil + M.M. Soil + mature mustard.

C. Control.

breaking up of the more complex nitrogen compounds. After 4 days this class of compounds begins to be broken up again, giving rise at the same time to ammonia. A little later nitrification sets in and nitrates begin to accumulate. The late nitrification is mainly due to the large accumulation of ammonia which checks the activities of nitrifying organisms for a time. With the increase of nitrates there is a sharp fall of ammonia and this goes on to the end of the period of incubation. At the same time the fall of the nitrogen oxidisable by hydrogen peroxide continues though more gradually than before. The water-soluble protein nitrogen remains almost constant at a very low level after the first rapid consumption (Fig. 8).

Mature mustard differs considerably in its chemical composition from the other three plant materials. Hence the nature of changes taking place in its nitrogen is strikingly different. It does not lose any appreciable amount of total nitrogen. There is a small but definite increase in the amount of total H_2O_2 nitrogen after the first 15 days, unlike the other green manures. This increase in nitrogen oxidisable by hydrogen peroxide is very probably due to the activities of the micro-organisms breaking down the hemicelluloses and cellulose which are present in larger quantities in this plant material than in the other three. The lack of available nitrogen is shown by the depression caused in the level of ammonia and nitrates below that of the control soil. In other words the micro-organisms are utilising the soluble nitrogen present in the soil for the decomposition of the soluble carbohydrates and the hemicelluloses. Hence there is no accumulation of ammonia nor of nitrates over a period of 65 days.

To sum up, the results presented in this series of experiments show that when a green manure rich in nitrogen is incorporated with the soil, large losses of nitrogen take place. The loss of nitrogen depends not only on the amount of total nitrogen contained in the green manure but also, and more especially, on the amount of nitrogen that is easily available. The greater the amount of total and available nitrogen the greater is the loss of nitrogen likely to occur. The results also give an insight into the nature of the changes taking place in the nitrogenous compounds of the green manures. The soluble nitrogenous compounds are attacked first and are metabolised into a more complex form of protein. This protein together with that existing in the green manure is then attacked and converted first into the less complex forms (*e.g.* water-soluble) and subsequently or simultaneously into ammonia and nitrates. Since a green manure rich in nitrogen is almost always comparatively poor in

carbohydrate compounds, there is the risk of a part of the ammonia being lost.

SUMMARY.

Four plant materials of widely different origin and age were used as green manures mixed with soil for decomposition studies under laboratory conditions.

Provided the conditions of temperature, moisture, aeration, and micro-flora, are optimal, the decomposition depends upon the chemical constituents of the plant materials. It is shown that the soluble carbohydrates, hemicelluloses and cellulose are the compounds mainly responsible for the loss of total organic matter during decomposition.

Plant materials containing a balanced proportion of available carbohydrate compounds to available nitrogenous compounds decompose rapidly. Those containing excess of nitrogenous compounds decompose more rapidly and those containing excess of carbohydrate compounds decompose less rapidly. This is true in all cases whether the plant material is a legume or a non-legume. Young plant materials by virtue of their abundance of available nitrogenous compounds decompose more quickly than mature tissues.

When comparatively young plant materials are used as green manure, there is the danger of a loss of nitrogen, the loss depending upon the amount of total and available nitrogen this contains. Not only do they lose nitrogen but they decompose very rapidly, with the result that nitrates accumulate soon after burial. Unless the succeeding crop is sown sufficiently early to utilise these nitrates, they are likely to be lost through leaching under field conditions.

ACKNOWLEDGMENT.

The author wishes to acknowledge his indebtedness to Sir John Russell for placing at his disposal the facilities of the Rothamsted Experimental Station and to the Authorities of the Bombay University for the award of the Eduljee Dinshaw Scholarship D during the tenure of which the work described in this paper was carried out.

He further takes this opportunity to accord his sincere thanks to Mr E. H. Richards and Dr H. H. Mann for their unfailing interest, guidance and advice, and to Drs A. G. Norman, E. M. Crowther, and S. H. Jenkins for discussions and criticism.

REFERENCES.

- (1) A.O.A.C. *Methods of Analysis* (1921), 2nd ed.
- (2) DAJI, J. A. *Biochem. J.* (1932), **26**, 1275.
- (3) HUTCHINSON, H. B. and RICHARDS, E. H. *J. Min. Agric.* (1921), **28**, 398.
- (4) NORMAN, A. G. *Biochem. J.* (1929), **23**, 1353.
- (5) — *Biochem. J.* (1929), **23**, 1367.
- (6) RANKER, E. R. *J. Assoc. Off. Agric. Chem.* (1927), **10**, 230.
- (7) REGE, R. D. *Ann. App. Biol.* (1927), **14**, 1.
- (8) SMITH, N. R. and HUMFIELD, H. J. *Agric. Res.* (1930), **41**, 97.
- (9) STEPHENSON, R. E. *Soil Sci.* (1918), **6**, 413.
- (10) — *Soil Sci.* (1921), **12**, 145.
- (11) TENNEY, F. G. and WAKSMAN, S. A. *Soil Sci.* (1929), **28**, 55.
- (12) WAKSMAN, S. A. *Principles of Soil Microbiology* (1927). Ballière, Tindall and Cox, London.
- (13) WAKSMAN, S. A. and DIEHM, R. A. *Soil Sci.* (1931), **32**, 119.
- (14) WAKSMAN, S. A. and TENNEY, F. G. *Soil. Sci.* (1927), **24**, 317.
- (15) — — *Soil Sci.* (1928), **26**, 155.
- (16) WHITE, J. W. *J. Agric. Res.* (1918), **13**, 171.
- (17) WHITING, A. L. and RICHMOND, T. E. *Soil Sci.* (1927), **24**, 31.
- (18) ZOLCINSKI, J. *Rocz. Nank. Poln. i. Lesnych* (1927), **17**, 349. (*Chem. Abs.* **22**, 3725.)
- (19) ZOLCINSKI, J. and MUSIEROWICZ, A. *Rocz. Nank. Poln. i. Lesnych* (1927), **17**, 377. (*Chem. Abs.* **22**, 3725.)

(Received June 1st, 1933.)

[*Reprinted from the Journal of Agricultural Science*, Vol. XXIV. Part I.]
[*All rights reserved.*]

THE FERTILISING VALUE AND NITRIFIABILITY OF HUMIC MATERIALS PREPARED FROM COAL.

By E. M. CROWTHER AND WINIFRED E. BRENCHELEY.

(Chemistry and Botany Departments, Rothamsted Experimental Station, Harpenden, Herts.)

(With Three Text-figures.)

LITTLE is known about the direct or indirect effects of the highly decomposed organic matter of the soil—the humic matter—on the nutrition and growth of cultivated crops. When present in considerable amounts it improves the physical properties of the soil by giving a better crumb structure and by increasing the water-holding capacity; its colloidal properties may influence the retention and availability of plant nutrients. The frequent use of heavy dressings of dung effects an improvement in soil fertility which is perceptible for many years, over fifty in the barley experiments at Rothamsted, and it is common knowledge among farmers and market gardeners that the fertility, especially of light land, falls off unless steps are taken to maintain the supply of soil organic matter. The materials available, crop residues and animal manures, are not inert sources of humic material. They also contain plant nutrients and readily oxidisable compounds which may profoundly modify the activities of the soil micro-organisms. It is almost impossible in such mixtures to assess the relative importance of the several components. Direct experiments on the addition of humic matter have rarely been attempted on account of the difficulty of preparing sufficient amounts of suitable material; indirect experiments⁽¹⁾ on the removal of humic matter necessarily cause profound changes in the composition of the soil.

The addition of peat has uncertain effects, at any rate in amounts suitable for ordinary arable soils, though it may have value for certain branches of horticulture. But peat cannot be regarded as providing a material akin to the humic matter of soil, for the peat necessarily remains in isolated lumps which become disseminated through the soil extremely slowly. Just as lumps of mineral phosphate are almost useless whereas superphosphate or very finely ground mineral phosphate may be useful as sources of available phosphorus in the soil, so materials which are expected to add directly to the humic matter of the soil should be

tested in the form either of very fine powders or as soluble salts. In recording negative results with a series of commercial humic materials, derived from brown coal or peat, O. Lemmermann⁽²⁾ mentioned that some promising but inconclusive results were obtained in field trials with an ammonium humate prepared from a peat extract. The preparation was however much too expensive to justify any commercial development which would allow tests on an adequate scale.

The desire to discover new outlets for coals of low industrial value has led to much discussion in the technical press of Central Europe on the possible value of brown coal and of certain humic materials prepared by the gentle oxidation of coal. It is possible to produce humic acids which give readily soluble ammonium or potassium salts, and it would appear that such products are more suitable for direct tests on the possible fertiliser value of humic materials than any of the cruder materials previously available. It has been claimed that there is already sufficient evidence to show that manuring with coal will become important in the near future. Unfortunately it would appear that many of the experiments have been made by workers who are more familiar with coal technology than with agricultural experimentation or soil chemistry. The statement of results sometimes suggests propaganda rather than research, and the discussions of the possible mechanism of the crop increases observed are unconvincing. They mention among others the stimulation of the growth of *Lemna* in water cultures, the improvement in physical properties, the direct assimilation of carbon compounds by plants, and the steady liberation of carbon dioxide and available nitrogen⁽³⁾. Apart however from the hypothetical stimulation of crop growth there is the possibility that a humic acid from coal might provide a suitable carrier for ammonia with certain advantages over ammonium sulphate or might be used with advantage as a drier in mixing compound fertilisers.

With the co-operation of A. Boake, Roberts & Co. Ltd., Stratford, E. 15 and The Powell Duffryn Steam Coal Company, Ltd., Cardiff, it has been possible to carry out a series of experiments in the field, the pot-culture house and the laboratory on humic acids (and their ammonium and potassium salts) prepared from finely ground coal by oxidation with nitric acid at 100° C. in a process in which the excess oxides of nitrogen are recovered for re-use⁽⁴⁾. The results showed that the ammonium in ammonium humate was as efficient as that in ammonium sulphate, and there was some indication that the humic material also supplied a small amount of available nitrogen. There was no evidence of any stimulation

158 *Value of Humic Materials Prepared from Coal*

or other beneficial effect apart from the production of ammonia and nitrate.

In any nitrogenous manure it is clearly essential to ascertain and allow for the availability of the nitrogen before proceeding to examine other effects. The experiments were therefore planned in such a way as to compare the availability of the nitrogen as determined by chemical analysis with the effects of the manures on the yield of the experimental crop. The fact that good correlations were obtained not merely disposes of hypothetical stimulations under the conditions of these experiments but shows that the laboratory technique for determining the availability of the nitrogen in the manure was adequate for its purpose.

The terms "Humic Acid" and "Ammonium Humate" will be used throughout this paper for convenience without, of course, implying that they are regarded as identical with materials present in or extractable from soils.

THE HUMIC MATERIALS.

The humic acid materials were dry fine grey-brown powders, and the ammonium humates were black coarse powders with bright surfaces. Some of the ammonium humates (*e.g.* the material *B* below) dissolved readily and completely in water to give stable dark brown solutions

Table I. *Percentage composition of the humic materials.*

		Dry matter	Am- monia N	Or- ganic N	Total N	Ash	P ₂ O ₅	K ₂ O
1930 samples:								
Humic acid	<i>E1*</i>	93.2	0.13	4.38	4.51	3.04	0.002	0.044
Ammonium humate	<i>F1*</i>	93.8	3.55	5.86	9.41	3.69	0.007	0.107
" "	<i>F2</i>	88.1	3.21	5.80	9.01	—	—	—
Potassium humate	<i>G1*</i>	85.1	—	3.36	3.36	—	—	16.3
			Ammonia N	Organic N	Total N			
1932 samples:								
Humic acid	<i>A1*</i>		—	4.43	4.43			
" "	<i>A2</i>		—	4.29	4.29			
" "	<i>A3</i>		—	4.24	4.24			
Ammonium humate (prepared from humic acid <i>A</i>)	<i>B1*</i>		3.16	5.14	8.30			
" "	<i>B2</i>		3.02	5.14	8.16			
" "	<i>B3</i>		2.87	5.13	8.00			
" "	<i>B4</i>		2.76	5.27	8.03			
" "	<i>B5</i>		2.88	5.07	7.95			
Humic acid	<i>C1</i>		—	3.82	3.82			
Ammonium humate (prepared from humic acid <i>C</i>)	<i>D1*</i>		1.24	4.72	5.96			

* Materials used in flask and pot experiments.

similar in appearance and general properties to alkaline humus extracts from soils. All of the humic materials used were prepared by the two firms mentioned. Several batches were used throughout the experiments, but it will be seen from Table I that the different preparations from the same initial materials remained substantially constant in composition. Humic acid *A* in 1932 and humic acid *E* in 1930 were from similar coals; humic acid *C* in 1932 was made from a different type of coal and its ammonium salt (*D*) contained much less ammonia than those (*B* and *F*) derived from the more normal materials. It will be noted that the humic materials contain much more nitrogen than normal coals and further that in the conversion of humic acid into ammonium humate the amount of organic nitrogen increases. A considerable fraction of the nitrogen used as catalyst in the oxidation of coal enters the humic acid. Some of the ammonia used to make the ammonium humate is combined as a product incapable of giving off ammonia on boiling with magnesia according to the methods of analysis laid down in the Regulations of the Fertiliser and Feeding Stuffs Act. In assessing the possible commercial value of such materials it must therefore be remembered that a considerable fraction of the organic nitrogen is derived from inorganic sources in the course of manufacture and that such nitrogen cannot therefore be produced more cheaply than that in the standard nitrogenous fertilisers. Before it is possible to assess any special properties of these materials it is essential to compare them against simple forms of nitrogen on the basis, first, of equal ammonia contents and, second, of equivalent total nitrogen contents. It is clearly recognised that organic materials may through their slower rate of decomposition and through indirect effects on the physical properties and microflora of the soil have effects which operate so slowly that they may be missed in short-period tests in laboratory, pot culture, or field experiments, but new nitrogenous fertilisers must be judged primarily by their immediate effect and, in any case, these immediate effects must be understood before satisfactory long-range experiments can be devised.

The experiments in the present paper were planned to secure as wide a range of experimental conditions as was possible in the course of a single season. A preliminary series of laboratory and pot-culture experiments was carried out in 1930 on a single type of soil. In 1932 similar experiments were made on four contrasting soil types and a series of field trials was conducted on several crops at a number of centres.

LABORATORY AND POT-CULTURE EXPERIMENTS, 1929.

The soil was taken from the side of Broadbalk Field, Rothamsted, but proved to be fundamentally different from the soil of the adjoining permanent wheat plots in that it was devoid of calcium carbonate. It was acid and, in addition, rich not only in nitrate but in readily nitrifiable material. Mustard sown in pots containing 10 kg. of soil on June 27th and thinned to three plants per pot was harvested on August 16th. There were eight treatments in quadruplicate. A basal dressing supplied potassium and phosphorus, the former being adjusted to allow for that in the potassium humate. The organic materials were used at the rate of 0.4 gm. of added total nitrogen per pot which corresponded with 0.15 gm. of nitrogen per pot as ammonia for the ammonium humate. Other pots therefore received 0, 0.15 and 0.40 gm. of nitrogen as ammonium sulphate. Soluble humates and insoluble humic acid were compared by the pairs (a) potassium humate and humic acid (both with and without added ammonium sulphate), (b) ammonium humate with humic acid and ammonium sulphate. The data in Table II show that the response of the mustard to ammonium sulphate was sufficient to provide a satisfactory range for testing different amounts of available nitrogen. The yield responses to humic acid (4 per cent. without and 5 per cent. with added ammonium sulphate) and to potassium humate (12 per cent. without and 2 per cent. with added ammonium sulphate), though consistently positive are too small to be regarded as significant individually, as is also the slight superiority of the ammonium humate over ammonium sulphate supplying equal ammonia. The experiment suggests but fails to establish a slight benefit from the humic materials as a whole, but it is clear that the immediate effect of an ammonium humate fertiliser must be ascribed to its ammonia rather than to its organic nitrogen or to any secondary effects of the humic material. Humic acid proved at least as efficient as sulphuric acid as an ammonia carrier.

The laboratory nitrification experiments were made simultaneously with the same soil and the same rates of addition of nitrogen (15 and 40 mg. per kg. soil). Large duplicate flasks were set up for each treatment and kept well aerated by frequent shaking. Analyses for ammonia and nitrate nitrogen were made after suitable intervals. The agreement between duplicate flasks was not sufficient to make it possible to detect small differences in response to treatment, but the results are in general agreement with those of the pot cultures. About 90 per cent. of the ammonium sulphate nitrified and only about a third of the organic nitrogen.

Table II.

Treatment	Un- treated	Humic acid <i>E</i>	Potassium humate <i>G</i>	Ammonium sulphate	Ammonium sulphate + humic acid <i>E</i>	Ammonium sulphate + potassium humate <i>G</i>	An- monium humate <i>F</i>	Ammonium sulphate	Standard error
N added in gm. per pot with 10 kg. soil:									
As ammonia N	—	—	—	0.15	0.15	0.15	0.15	0.40	—
As organic N	—	0.4	0.4	—	0.25	0.25	0.25	—	—
As total N	—	0.4	0.4	0.15	0.40	0.40	0.40	0.40	—
Pot experiments:									
Mustard, dry matter in gm. per pot	14.6	15.1	16.1	18.4	19.0	18.6	18.8	21.1	0.68
Percentage increase over untreated pot	—	4	12	26	31	28	29	46	6.6
Flask experiments:									
Ammonia N in mg. per kg. soil after days:									
1	10	9	10	—	20	—	19	43	—
21	2	3	3	—	5	—	4	12	—
49	2	1	1	—	2	—	2	4	—
Nitrate N in mg. per kg. soil after days:									
1	33	31	34	—	32	—	32	33	—
7	55	55	54	—	53	—	55	56	—
14	62	58	60	—	62	—	67	74	—
21	67	57	59	—	65	—	66	79	—
49	68	66	71	—	69	—	74	94	—

162 *Value of Humic Materials Prepared from Coal*

It will be noted that although nitrification was relatively slow in this experiment and much ammonia remained from ammonium sulphate even after three weeks, yet there was no evidence of any accumulation of ammonia from the humic materials.

LABORATORY EXPERIMENTS, 1932.

The 1932 experiments tested two humic acids and their ammonium humates on four soil types selected so as to give as wide a range of conditions as possible.

Table III. *Mechanical analyses of soils from pot-culture and flask experiments 1932. Weights of oven-dry fractions as percentages of air-dry soil.*

	Soil			
	R.	S.	T.	W.
Coarse sand	20.3	33.7	58.5	50.1
Fine sand	37.9	28.4	28.6	29.1
Silt	22.5	10.2	5.3	7.5
Clay	15.0	21.5	5.5	10.0
Carbonates	0.0	0.9	0.0	0.0
Moisture	2.2	2.5	0.6	1.1
Loss on solution	1.0	0.8	0.4	0.5
Difference of sum from 100	1.1	2.0	1.1	1.7
Loss on ignition	5.3	4.6	1.0	3.0
pH	5.0	7.4	5.0	5.8

The four soils, whose mechanical analyses of the soils are given in Table III, were:

R. (Rothamsted). As in the 1930 experiments, the soil was taken from the side of Broadbalk Field. It was an acid heavy loam probably containing organic matter remaining from the time some decades ago when the broad headlands of the field were kept under grass.

S. (Saxmundham). A calcareous clay loam from calcareous boulder clay at the Saxmundham Experimental Station, E. Suffolk.

T. (Tunstall). A light acid sand from the Tunstall Experimental Station, E. Suffolk.

W. (Woburn). A slightly acid sandy loam from drift on Lower Greensand at the Woburn Experimental Farm, Beds.

The conditions of the nitrification experiments were standardised much more rigidly than in the preliminary 1930 series. 50 gm. lots of air-dry soil were set up in 350 c.c. conical flasks with the appropriate amount of humic material or ammonium sulphate to give 15 or 40 mg.

of added total nitrogen per kilogram of soil in the series of treatments given in Table IV.

Table IV.

Material added	Nitrogen added in mg. per kg. soil		
	As ammonia	Organic	Total
(1) None	0	0	0
(2) Humic acid <i>A</i>	0	40	40
(3) Ammonium humate <i>D</i> (prepared from humic acid <i>C</i>)	8	32	40
(4) Ammonium sulphate	15	0	15
(5) Ammonium humate <i>B</i> (prepared from humic acid <i>A</i>)	15	25	40
(6) Ammonium sulphate + humic acid <i>A</i>	15	25	40
(7) Ammonium sulphate	40	0	40

Each of the seven treatments was given to six independent flasks for each soil and one flask was taken for analysis after 2, 4, 6, 8, 17 or 26 weeks. The soils were brought to about optimal water content, all of the flasks of one soil receiving equal measured amounts, and the flasks were loosely plugged with cotton-wool and kept in a cellar with little fluctuation of temperature. The flasks were aerated by vigorous shaking every few days and adjusted to constant weight every week by the replacement of the water lost by evaporation. Except in the Saxmundham soil, a satisfactory crumb condition was maintained throughout the experiment. Unfortunately the soils were allowed to become too dry shortly before the analysis at 17 weeks, and the relatively large amount of water added was not uniformly distributed before analysis. This caused an unusual irregularity in the results at this time and also caused the heavy Saxmundham soil in one of the last lot of flasks to gather together into large balls.

After the appropriate period of incubation one flask for each soil and each treatment was analysed for ammonia and nitrate by Carsten Olsen's method⁽⁵⁾. The whole of the soil was shaken for 1 hour with *N* KCl containing enough HCl to give a final *pH* of about 1.0. After filtering an aliquot part was distilled with magnesia and then with Devarda's alloy. The initial ammonium and nitrate contents of the soils as set out in Table III were obtained from analyses on the untreated soils, together with the amounts of ammonia added in the various treatments.

This technique has the advantage that it eliminates sampling errors other than those involved in setting up the flasks. Every analytical result is independent of and strictly comparable with every other one.

164 *Value of Humic Materials Prepared from Coal*

The data include all of the irregularities and errors not merely of the initial sampling and of the analysis but also of the growth and activities of the micro-organisms throughout the whole experiment. The separate flask technique is greatly to be preferred to that used in the preliminary 1930 series and in much older work in which a few large samples of soil were set up and small samples drawn at the required intervals. Evidence has often been obtained in these laboratories to show that aeration and other irregularities introduced by sampling from a mass of soil may seriously disturb the course of the microbiological activity and the amounts of ammonia and nitrate accumulated.

There are of course no independent replicates, but there can be little objection to taking a high order interaction between treatment, time, and soil as an estimate of error. The following analyses of variance for total inorganic nitrogen in mg. per kg. soil for the periods 2, 4, 6, 8 weeks and for 2, 4, 6, 8, 17, 26 weeks show that the error so estimated is satisfactorily low, especially in the earlier periods before the moisture irregularity already mentioned.

	Period, 2, 4, 6, 8 weeks		Period, 2, 4, 6, 8, 17, 26 weeks	
	Degrees of freedom	Sum of squares	Degrees of freedom	Sum of squares
Soils	3	63,843	3	126,638
Treatment	6	13,836	6	19,857
Time	3	626	5	4,653
Interactions:				
Soils \times time	9	1,071	15	6,394
Soils \times treatment	18	119	18	296
Time \times treatment	18	108	30	410
Soils \times time \times treatment	54	181	90	735
Total	111	79,783	167	158,982
Standard error per flask		1.83		2.86
General mean		42.6		45.0
Standard error per flask as percentage of mean		4.3		6.4

This analysis of variance shows that the differences between soils, between treatments and for different times are all highly significant. The first-order interactions between these factors taken in pairs are of much smaller magnitude. The interaction of soils and time is definitely significant or, in other words, some soils accumulate inorganic nitrogen more rapidly than other soils, quite irrespective of fertiliser treatment. The nature of this effect is shown in Figs. 1*a* and 1*b* (for the analyses at 2, 4, 6 and 8 weeks), from which it will be seen that when the treatments are averaged there is a great and steady accumulation of nitrate and a slow loss of ammonia from the Rothamsted soil and similar but much

smaller effects in the Tunstall soil. Both of these soils are appreciably acid. In the other two soils the ammonia disappears more rapidly and the total inorganic nitrogen remains substantially constant. When fertiliser treatments are considered as a whole their interaction with soils is quite small and only on the verge of significance, whilst that with time is significant.

Table V gives the inorganic nitrogen contents for the seven fertiliser treatments at each time of analysis, averaging the four soils, and the mean recovery of added nitrogen (excess for treated over untreated soil) for each of the soils, averaging the analyses from 2 to 26 weeks. Fig 1c

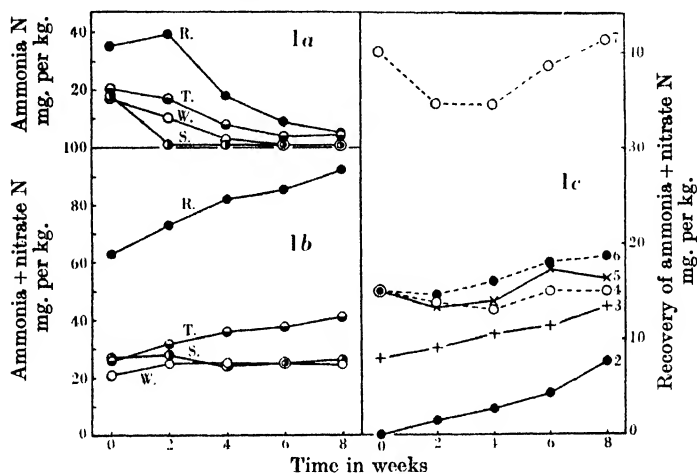


Fig. 1. Nitrification experiments on four soils. Treatments and soils as in Table V. *a*. Ammonia contents of four soils (averaging seven fertiliser treatments). *b*. Ammonia + nitrate contents of four soils (averaging seven fertiliser treatments). *c*. Mean recovery of ammonia + nitrate for seven fertiliser treatments (averaging four soils).

gives the mean recovery of added nitrogen for the analyses at 2, 4, 6, 8 weeks, averaging the four soils. It will be noted that there is a slight progressive accumulation of inorganic nitrogen from humic acid *A* and from ammonium humate *D*. The progressive accumulation of inorganic nitrogen from humic acid *A* varies from soil to soil, and this effect accounts for much of the observed interaction between soils and treatment and between time and treatments. It is examined in more detail below. With these minor qualifications the relative unimportance of soil and time effects shows that the nitrifiabilities of the six fertiliser materials may safely be expressed by the mean inorganic nitrogen productions, averaging all time of analysis and all soils. Conclusions from the means of four soils, all showing similar effects, are clearly of more general value

Table V. *Ammonia and nitrate accumulation in incubated soils. All results as mg. of N per kg. soil (parts N per million).*

Treatment		1	2	3	4	5	6	7
		None	Humic acid A	Ammonium humate D	Ammonium sulphate	Ammonium humate B	Ammonium sulphate + humic acid A	Ammonium sulphate
Ammonia N added		—	—	8	15	15	15	40
Total N added		—	40	40	15	40	40	40
N found as $\text{NH}_3 + \text{NO}_3$ (averaging 4 soils) (standard error = 1.43):								
Initially	21	21	21	29	36	36	36	61
After 2 weeks	27	29	29	35	41	40	42	62
4	29	32	32	38	42	44	45	63
6	28	33	33	39	44	46	46	67
8	30	38	38	42	45	46	49	72
17	31	33	33	34	45	46	52	66
26	42	48	48	54	54	58	59	75
Recovery of added N as $\text{NH}_3 + \text{NO}_3$ (averaging six times of analysis for 2 to 26 weeks) (standard error = 1.65):								
(a) Excess over untreated soil:								
Rothamsted soil	—	6	12	15	18	18	21	40
Saxmundham soil	—	6	10	14	14	14	18	33
Tunstall soil	—	2	7	14	14	16	16	35
Woburn soil	—	2	8	13	13	14	15	38
(b) Excess over soil with 15 mg. N as ammonium sulphate:								
Rothamsted soil	—	—	—	—	—	4	6	25
Saxmundham soil	—	—	—	—	—	0	4	19
Tunstall soil	—	—	—	—	—	3	2	21
Woburn soil	—	—	—	—	—	0	2	24

than those which would have been obtained if a similar number of analyses had been devoted to replicated tests on a single soil. The similarity of the results in four soils also suggests that nitrate accumulation depends on the availability of the original materials rather than on secondary effects dependent on hypothetical stimulations or on improvements in physical conditions. The mean recoveries of inorganic nitrogen from the added materials are given in Table VI.

Table VI. *Recovery of inorganic nitrogen (in mg. per kg. soil).*

Treatment	Ammonia N added	Total N added	Inorganic N recovered (mean for 2-26 weeks and for 4 soils)
(2) Humic acid <i>A</i>	0.0	40.0	4.1
(3) Ammonium humate <i>D</i>	8.4	40.0	9.0
(4) Ammonium sulphate	15.2	15.2	13.9
(5) Ammonium humate <i>B</i>	15.2	40.0	15.5
(6) Ammonium sulphate + humic acid <i>A</i>	15.2	40.0	17.4
(7) Ammonium sulphate	40.0	40.0	36.2
Standard error	—	—	0.82

The initial ammonium content is clearly the primary factor in determining the amount of nitrate formed from the fertilisers. Only small and insignificant amounts of the organic nitrogen of the ammonium humates were nitrified, but there was a small but definite accumulation of nitrate from the humic acid both in the presence and in the absence of added ammonium sulphate. By averaging the two humic acid series (neglecting the difference in the amounts taken for treatments 2 and 6) the nitrification of humic acid may be examined in more detail. In all there were 48 comparable pairs of flasks with and without humic acid *A* and in 43 of these pairs there was more nitrate in the flask with humic acid. This fact alone is sufficient to demonstrate that humic acid caused some nitrate formation.

Table VII. *Recovery of N (in mg. per kg. soil) as NH₃ and NO₃ from humic acid A. Mean effect of humic acid in presence and absence of 15 mg. N as ammonium sulphate.*

Soil	Time in weeks					
	2	4	6	8	17	26
Rothamsted	1	4	4	5	10	10
Saxmundham	2	3	5	8	4	8
Tunstall	1	0	3	3	4	1
Woburn	0	4	2	4	-1	3
Mean difference between heavy (R. + S.) and light (T. + W.) soils	1.3	1.8	2.3	3.0	5.2	7.2

The results given in Table VII show that the extra nitrated due to humic acid is greater in the two heavy soils than in the two lighter ones, and further that the excess of nitrate production from humic acid in the heavy over that in the light soils increases most regularly with time. This differential effect of heavy and light soils on the rate of nitrate accumulation from humic acid accounts for the greater part of the interactions between treatment and soil and between treatment and time already mentioned. Its regular change with time forms but one of the 90 degrees of freedom in the highest order of interaction used for estimating the laboratory error, but separating it from the other 89 degrees would have only a trivial effect on this estimate of error. The increased nitrate from humic acid, especially in heavy soils, may be due not merely to a direct nitrification of its organic nitrogen but to improved physical conditions, such as better crumb structure, leading to a more complete oxidation of the soil organic matter. The fact that the effect is associated with heaviness rather than with soil reaction or reserves of nitrifiable material would point to this indirect effect but it is naturally impossible to decide the mechanism from these experiments alone.

POT-CULTURE EXPERIMENTS, 1932.

Pot cultures with barley followed by mustard were carried out in quadruplicate on the four soils and with the seven manurial treatments used in the flask experiments. Each pot contained 10 kg. of soil and ammonium sulphate was given at the rate of either 0.152 or 0.400 gm. of nitrogen per pot. The organic manures throughout supplied 0.400 gm. of nitrogen per pot (the ones with humic acid and ammonium sulphate had 0.4 gm. nitrogen and 0.152 nitrogen respectively in these forms and not a total of 0.400 gm. nitrogen as in the laboratory experiments). Each pot had a basal dressing of 2 gm. of dipotassium hydrogen phosphate. A pedigree straw of Goldthorpe barley, graded to 0.05–0.06 gm. per seed, was sown on March 31st, 1932, at the rate of six seeds per pot and thinned out on April 4th to three plants per pot. The ears and straw of each pot were oven dried and weighed and the produce of replicate pots bulked for threshing and nitrogen determinations.

After the barley harvest the soil of each pot was thoroughly remixed and a further 2 gm. of potassium phosphate added. White mustard (*Brassica alba*) was sown on August 22nd, thinned on September 8th to three plants per pot, and harvested on October 28th. All mustard yields were low and generally large barley crops were followed by small mustard crops. The growth of the barley and the decomposition of its residues so

completely exhausted the supply of available nitrogen that they masked any residual effect from the relatively slow decomposition of the organic manures. In fact the excess of nitrogen in the manured crops over that in the unmanured ones was less for the two crops, barley and mustard, than for the first alone.

The yields of dry matter in barley grain and barley straw are plotted in Fig. 2 against the mean nitrogen recoveries from the same treatments in the laboratory nitrification experiments (a slight correction was made in treatment 6 for the different amounts of humic acid used in the two

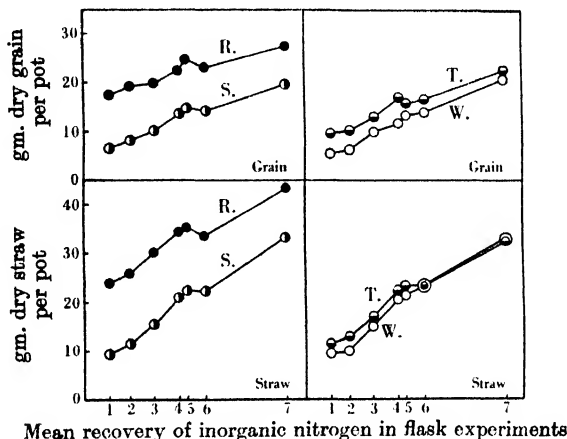


Fig. 2. Grain and straw yields in gm. dry matter per pot for seven fertiliser treatments in four soils, plotted against mean recovery of inorganic nitrogen in flask nitrification experiments (averaging four soils and all times of analysis) (Table VI).

series). For all soils there was a sufficient range of yields to reveal differences in the efficiencies of the fertilisers. The Rothamsted soil gave much higher yields than the others.

The effects due to fertilisers were so closely similar in the four soils that it is unnecessary to tabulate the values for individual soils. The discrepancies between the relative effects in the four soils (*i.e.* the interaction between soils and treatments) may be used to provide a standard error with which to test the average response to treatment when the four soils are averaged. Table VIII gives the yields, nitrogen percentages, and total nitrogen contents of the crops averaging, first, the four soils and, second, the seven treatments. As in the nitrification experiments the effects of the ammonium humates are never significantly above those of ammonium sulphate equivalent to the ammonium of the ammonium humates. There is thus no definite evidence of any benefit from the humic

Table VIII. *Yields and nitrogen contents (in gm. per pot of 10 kg. soil) for barley grain (G), barley straw (S.) and mustard (M.) in pot cultures in four soils.*

Results for 7 treatments, averaging 4 soils:

	Nitrogen added		Yield of dry matter			Nitrogen % in dry matter			Total nitrogen in crops			
	NH ₃	Total	G.	S.	M.	G.	S.	M.	G.	S.	G. + S.	G. + S. + M.
(1) Untreated	—	—	9.7	13.6	3.67	1.40	0.305	2.40	0.138	0.042	0.180	0.267
(2) Humic acid A	—	0.400	10.8	15.1	3.69	1.38	0.314	2.38	0.151	0.048	0.199	0.287
(3) Ammonium humate D	0.083	0.400	13.1	19.5	2.29	1.22	0.291	2.46	0.165	0.058	0.223	0.303
(4) Ammonium sulphate	0.152	0.152	16.3	24.8	2.91	1.22	0.286	2.47	0.200	0.072	0.273	0.344
(5) Ammonium humate B	0.152	0.400	17.1	25.7	2.97	1.22	0.288	2.56	0.211	0.075	0.286	0.360
(6) Ammonium sulphate + humic acid A	0.152	0.552	16.9	25.8	3.11	1.24	0.298	2.48	0.211	0.077	0.288	0.365
(7) Ammonium sulphate	0.400	0.400	22.7	35.6	2.94	1.40	0.360	2.42	0.320	0.129	0.448	0.519
Standard error			0.48	0.51	0.139	0.025	0.0069	0.052	0.0048	0.0018	0.0057	0.0072

Results for 4 soils, averaging 7 treatments:

Rothamsted soil	—	—	22.0	32.4	5.00	1.41	0.328	2.53	0.310	0.106	0.418	0.545
Saxmundham soil	—	—	12.3	19.5	3.83	1.25	0.273	2.18	0.167	0.054	0.207	0.290
Tunstall soil	—	—	15.0	20.5	2.38	1.31	0.301	2.44	0.191	0.063	0.258	0.316
Woburn soil	—	—	11.6	19.0	1.70	1.22	0.322	2.66	0.139	0.062	0.201	0.246
Standard error			0.36	0.38	0.105	0.019	0.0052	0.039	0.0036	0.0013	0.0043	0.0054

material either directly from its organic nitrogen or indirectly through any stimulation or improvement in the physical conditions of the soil. The increases due to humic acid are insignificant in yields of barley grain but just significant in the yields of straw and in the total nitrogen contents of the barley grain and straw and of the two crops. These small effects may be taken as suggesting that a little nitrogen became available relatively late in the growth of the barley. Apart from this small effect the general results for the nitrogen percentages of grain and straw show with increasing yields a progressive fall to a minimum and then a rise. This is evidence that the barley plants obtained most of their nitrogen at a

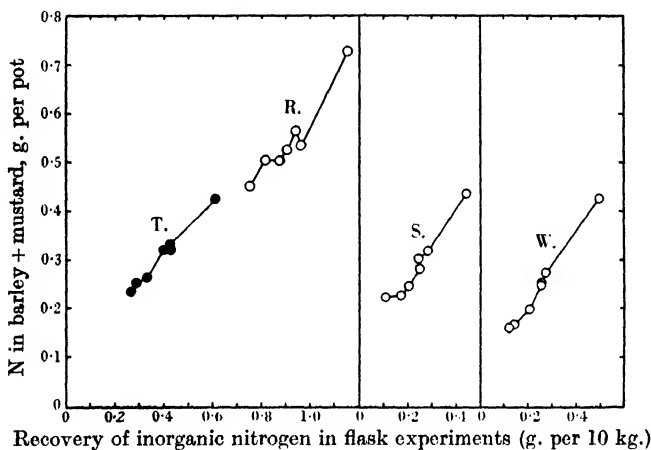


Fig. 3. Total nitrogen contents of barley, grain and straw, and mustard plotted against mean inorganic nitrogen content for the same soil and treatment calculated from laboratory nitrification experiments (averaging all times of analysis).

sufficiently early stage of growth to allow of its efficient utilisation for carbohydrate synthesis. Both in yield and composition the barley plants showed that they received rapidly available nitrogen in amounts almost proportional to the ammonium nitrogen added in the fertilisers; only for humic acid was there any suggestion of a slight production of slowly available nitrogen. The general conclusions are thus identical with those from the laboratory nitrification experiments.

Fig. 3 gives a detailed comparison for the four soils separately of the inorganic nitrogen found in flask experiments and that contained in the two crops of the pot experiments (the discrepancy between the amounts of humic acid used in treatment 6 in the two series is neglected). The points are joined in the order in which the fertiliser treatments are arranged in Table VIII. It is obvious that the technique for assessing

the relative availabilities of nitrogen in the fertilisers gave results closely parallel to the amounts absorbed by the crops in the pot cultures for comparisons in a single soil. This must be regarded as establishing the value of the laboratory technique and also as showing that for barley as grown in pot cultures the effects of the humic materials are entirely determined by the readily available nitrogen they supply. The fact that such results were obtained in four soils selected to secure the widest possible contrasts greatly increases the likelihood that the conclusions will be applicable to a wide range of soil conditions in the field. It must be concluded that the first year effects of such humic materials are to be assessed in terms of their ammonia contents with the possible qualification that humic acid *A* may act as a source of a little slowly available nitrogen.

FIELD EXPERIMENTS.

Field experiments were undertaken in 1932 to study the fertiliser value of ammonium humate *B* and humic acid *A* under as wide conditions of soils and crops as possible. The patterns of the experiments varied according to the local facilities and requirements. At Rothamsted and Woburn the experiments were on kale which was selected as a relatively slowly growing crop likely to utilise nitrogen over a long period. There were experiments on mangolds at Wye and Welshpool and on sugar beet at Wye and Tunstall. Four centres had potato experiments and three barley experiments on uniform plans.

Details of the previous cropping, layout and individual plot yields are given in the Rothamsted Report for 1932. Only summaries of the final yields and the relevant standard errors will be given here.

At Tunstall an experiment was made on sugar beet on a light sand to test ammonium humate supplying the same ammonia nitrogen as a standard dressing of ammonium sulphate (0.4 cwt. of nitrogen per acre). Over 1 ton of ammonium humate was required per acre. The test was made by splitting the 16 plots of a Latin Square experiment into humate and sulphate halves at random and was sufficiently sensitive to establish as significant a mean difference of only 2 per cent. in yield of roots, but the difference between the yields from the two manures fell below this amount.

The three barley experiments failed to reveal significant differences in grain yield between treatments when the experiments were considered individually, but the average of the three experiments showed that both sodium nitrate and ammonium sulphate increased the grain yields signi-

ficantly and sodium nitrate gave a significantly higher yield than ammonium humate with equal total nitrogen. Ammonium humate gave about half the yield given by ammonium sulphate. The mean recovery in the grain of added nitrogen was 23 per cent. for ammonium sulphate and 9 per cent. for ammonium humate. The yields of barley straw showed wider differences than those of grain. At two of the centres—Wellingore and Sparsholt—there were significant yield differences of the type shown by the average grain yields for the three.

Table IX. *Experiments on barley, 1932.*

	<i>O</i> No nitrogen	<i>B</i> Ammonium humate	<i>S</i> Ammonium sulphate	<i>N</i> Sodium nitrate	Standard error
Grain yield, cwt. per acre:					
Wye, Kent	27.1	27.8	30.0	29.8	1.32
Sparsholt, Hants	22.4	24.7	25.4	27.0	1.33
Wellingore, Lincs	29.0	29.9	30.9	30.7	0.96
Straw yield, cwt. per acre:					
Wye	32.0	33.2	38.5	38.8	2.10
Sparsholt	22.0	24.8	28.9	29.5	1.38
Wellingore	29.1	30.7	33.5	36.4	1.20
Nitrogen % of dry matter in grain:					
Wye	1.24	1.24	1.28	1.30	—
Sparsholt	1.31	1.32	1.38	1.37	—
Wellingore	1.50	1.53	1.60	1.70	—
Percentage recovery in gm. of added nitrogen:					
Wye	—	3	20	21	—
Sparsholt	—	13	23	31	—
Wellingore	—	10	26	36	—

Significant differences in straw yield: at Wye, $S=N>O$; at Sparsholt, $S=N>B>O$; at Wellingore, $S=N>O$; $\frac{1}{2}(S+N)>B$.

The potato experiments were on small plots and none of the differences attained the rather high levels required for significance.

The Wye sugar-beet experiment gave significant differences for the three levels of nitrogen as ammonium sulphate (0, 0.15 and 0.4 cwt. per acre) but failed to distinguish between ammonium humate and ammonium sulphate of equal ammonium content. The Wye experiment on mangolds proved to be the most sensitive of the series. Ammonium humate again failed to give higher yields than ammonium sulphate of equal ammonium content, in spite of the circumstance that the higher dressing of ammonium sulphate gave a further large increase in yield, but humic acid gave a significant increase over no nitrogen. At Welshpool however the addition of humic acid with ammonium sulphate reduced the yield of mangold tops below that from ammonium sulphate alone.

The kale experiments provide conditions somewhat approaching

Table X. *Experiments on root crops, 1932. All yield in tons per acre.*

Treatment	O	A	Ammonium				S2	N	Significant differences
			Humic acid	monium sulphate + humic acid	monium humate	Ammonium sulphate			
N added as NH ₄ or NO ₃ , cwt. per acre	None	—	—	0.15	0.15	0.15	0.4	0.4	—
Organic N added, cwt. per acre	—	0.40	—	—	0.25	0.25	—	—	—
Total N added, cwt. per acre	—	0.40	0.15	0.15	0.40	0.40	0.4	0.4	—
Centre									
Crop									
Wye, Kent	Mangolds	13.7	15.6	17.3	—	18.6	20.9	—	S2 > B = S1 > A > O
Welshpool, Mont.	Mangolds, roots	—	—	12.9	12.2	13.5	15.6	—	S2 > others
"	Mangolds, tops	—	—	5.6	4.5	5.2	6.2	—	S2 > S1 = B > S1 + A
Wye, Kent	Sugar beet, washed roots	12.0	—	12.7	—	12.8	13.8	14.0	N = S2 > B = S1 > O
"	Sugar beet, tops	7.6	—	8.3	—	8.4	9.6	10.4	N = S2 > B = S1 = O
Burford, Oxford	Potatoes	—	—	7.6	8.5	7.7	8.5	—	0.59
Tonbridge, Kent	Potatoes	—	—	10.0	10.0	10.2	9.8	—	0.38
Godalming, Surrey	Potatoes	—	—	10.1	9.1	10.6	10.2	—	0.32
Hull, Yorks	Potatoes	—	—	11.1	10.8	10.9	11.2	—	0.46

those of the market-garden crops for which slowly acting organic manures are highly favoured. The differences in yield and nitrogen contents were small. At Rothamsted but not at Woburn the heavier dressing of ammonium sulphate gave a significantly higher yield than the lower one showing that the crop was capable of responding to added nitrogen. There were, however, no responses to the organic forms of nitrogen. At Woburn humic acid gave a slightly lower yield and nitrogen content than the unmanured plots.

Table XI. *Kale experiments at Rothamsted (R.) and Woburn (W.), 1932.*

	O	A	S1	B	S2	
	No N	Humic acid	Ammonium sulphate	Ammonium humate	Ammonium sulphate	Standard error
N added as NH_4 , cwt. per acre	0	0	0.145	0.145	0.4	—
N added as organic matter, cwt. per acre	0	0.4	0	0.255	0	—
Fresh crop, tons per acre:						
R.	12.6	12.9	13.8	13.8	15.4	0.38
W.	17.6	16.9	18.8	18.0	18.8	0.62
Dry matter, tons per acre:						
R.	1.87	1.94	2.11	2.09	2.17	—
W.	2.96	2.82	3.12	2.97	3.08	—
N in crop, cwt. per acre:						
R.	0.65	0.69	0.69	0.72	0.79	—
W.	1.17	1.09	1.19	1.24	1.24	—
Percentage N recovery:						
R.	—	10	18	26	34	—
W.	—	(-20)	16	18	18	—

Significant results in fresh crop: at Rothamsted, $S2 > S1 = B > O$; at Woburn, none.

When the whole of the comparable results in the above series of experiments was averaged it was found that the slight superiority of ammonium humate over ammonium sulphate of equal ammonium content was too small to be regarded as more than fortuitous. The single significant positive result from humic acid must be regarded as offset by a negative result. The results for a wide range of soil and crops show that the fertilising value of these humic materials in a single season may be assessed from their ammonium contents as determined in the Regulations of the Fertiliser and Feeding Stuffs Act.

SUMMARY.

1. Humic acids and ammonium humates prepared by a patented process of gentle oxidation of coal were examined as fertilisers by laboratory nitrification experiments and pot-culture tests on four soils

and by a number of field experiments on a range of soils and crops during a single season.

2. In all tests the effects of ammonium humate could not be distinguished from those of ammonium sulphate of equal ammonium content. The nitrification tests and the pot cultures afforded some evidence of a slow production of nitrate or available nitrogen from the humic acid.

3. In the field experiments as in the pots there was no clear evidence of any fertiliser value apart from that due to the ammonium present.

4. The close agreement between laboratory measurements on nitrate accumulation and yields and nitrogen contents of barley for seven treatments in four soils shows that the laboratory technique afforded an adequate measure of the availability of the fertiliser nitrogen.

ACKNOWLEDGMENTS.

We wish to record our indebtedness to Mr F. McNaughtan of the Government Agricultural Chemistry Laboratory, Kenya, who carried out most of the 1932 laboratory work during a period of study leave, and to a number of local workers, whose names and addresses are given in the Rothamsted Report for 1932, for conducting the necessary field experiments under Rothamsted supervision.

REFERENCES.

- (1) WEIR, W. *J. Agric. Sci.* (1915), **7**, 246. CROWTHER, E. M. *J. Agric. Sci.* (1925), **15**, 303.
- (2) LEMMERMANN, O. *Z. Pflanz. Düng.* (1933), **30**, 1.
- (3) LIESKE, R. *Trans. Fuel Conference*, London (1928), **1**, 80; *Brennstoff-Chemie* (1929), **10**, 437; (1931), **12**, 81. KISSEL, A. *Brennstoff-Chemie* (1930), **11**, 257; (1931), **12**, 101, 245.
- (4) CARROLL, M. F. and A. BOAKE, ROBERTS AND CO. LTD. British Patents 361890 and 388134.
- (5) OLSEN, C. *Compt. rend. Trav. Lab. Carlsberg* (1929), **17** (15), 12.

(Received October 14th, 1933.)

STUDIES ON CALCIUM CYANAMIDE.

IV. THE USE OF CALCIUM CYANAMIDE AND OTHER FORMS OF NITROGEN ON GRASSLAND.

By H. L. RICHARDSON.

*(Chemistry Department, Rothamsted Experimental Station,
Harpenden, Herts.)*

AMONG the numerous investigations of nitrogenous fertilisers on grassland that have been reported in recent years, little attention has been given to differences in the action of widely different forms of nitrogen. Such differences might be expected to appear especially clearly with this crop, which remains in the ground throughout the year and completely occupies the soil with its roots. It has the additional advantage that repeated cutting is possible, giving samples over a lengthened growing period, and enabling a closer examination of the action of the nitrogen to be made.

Experiments with calcium cyanamide (the commercial form of calcium cyanamide, CaCN_2) were therefore made on pasture grassland, in order to examine its reputation for being "slow acting" in comparison with sulphate of ammonia. As it was possible that this slow action would allow the fertiliser to be applied in late autumn in order to secure an "early bite" in the spring, autumn and winter applications were especially studied. In the first experiment several forms of nitrogen applied in winter were compared with a spring dressing of sulphate of ammonia. This experiment was repeated in the following year, and in addition a direct comparison of calcium cyanamide and sulphate of ammonia was made in both autumn and spring.

In the experiments with winter nitrogen, two of the treatments contained some of their nitrogen as dicyanodiamide. This compound ($\text{NH}_2 \cdot \text{C}(:\text{NH}) \cdot \text{NH} \cdot \text{CN}$) is known to check nitrification in the laboratory, at concentrations which are not appreciably toxic to plants. It is formed when superphosphate is mixed with calcium cyanamide, to an extent which may be controlled by varying the conditions of mixing (cf. Richardson (1)). The substance therefore seemed a useful one to employ with the idea of reducing any loss of nitrogen due to nitrification followed by winter leaching. The dicyanodiamide used was prepared by boiling

calcium cyanamide with water, crystallising out and recrystallising. Its melting-point was 200–202° C. The writer is indebted to Dr E. H. Farmer for permission to use the Whiffen Laboratory at the Imperial College of Science and Technology for its preparation.

As in the earlier cyanamide investigations (2) soil analyses were made in addition to the studies of the plant. Nothing was known when the work was begun about the behaviour of added ammonia in grassland soils, and in this respect the results are not without general interest.

THE INORGANIC NITROGEN OF GRASSLAND SOILS.

(Stackyard Field experiment, 1929–30.)

Young grassland was chosen for this experiment; the soil, the heavy loam with flints of Rothamsted, having been sown with a seeds mixture in the spring of 1928 (for further details of management see later under "Herbage").

In comparison with the old grassland soil of the Rothamsted Park, the soil had more flints near the surface, and was stickier to handle but held less water in the winter. It nitrified more vigorously, *i.e.* it produced more nitrate and less ammonia on incubation. The soil was naturally well drained, and it had been chalked while under cultivation; its reaction was now pH 6.5. There was no "mat," and roots were numerous to the depth of sampling (8 in.).

The treatments compared were:

- (1) No nitrogen.
- (2) Winter sulphate of ammonia (0.4 cwt. nitrogen per acre).
- (3) Winter sulphate of ammonia (0.2 cwt. nitrogen) with dicyanodiamide (0.2 cwt. nitrogen).
- (4) Winter calcium cyanamide (0.4 cwt. nitrogen).
- (5) Winter calcium cyanamide (0.2 cwt. nitrogen) with dicyanodiamide (0.2 cwt. nitrogen).
- (6) Spring sulphate of ammonia (0.4 cwt. nitrogen).

The 0.4 cwt. per acre of nitrogen applied corresponds to 2 cwt. per acre of sulphate of ammonia or calcium cyanamide. This represents approximately 20 mg. of nitrogen per kg. of dry soil in the samples taken. There was sixfold replication of treatments, giving thirty-six plots which were sampled and analysed separately in two blocks on consecutive days.

In sampling the soil a semi-cylindrical tool of narrow bore was used, to

reduce the disturbance of the soil through repeated samplings. The depth of 8 in. was decided on after considering the results of an experiment with fallow during the previous winter (Crowther and Richardson (2)); it was approximately the depth of the old ploughed layer.

The general procedure was to take a dozen or more cores per plot, the sample from each plot (amounting to 150–200 gm.) being collected in a separate bottle. In the laboratory the soil was broken into fine crumbs, larger flint or vegetable fragments and roots being removed, and 100 gm. were taken for the preparation of an extract by the modified Carsten-Olsen method (Crowther and Richardson, *loc. cit.*). The extracts, containing toluene as a preservative, were always prepared on the day of sampling, the ammonia and nitrate distillations being carried out during subsequent days.

At first a separate 50 gm. subsample was taken from each plot sample for dry matter determination, but the standard error of the dry-matter values (0.43 per cent. of the weight of the samples, for a single plot) proved to be much lower than that of the mineral nitrogen. Subsequently, therefore, four to six composite samples were made and the dry matter was determined in these.

The complete sampling of the thirty-six plots was continued, usually at fortnightly intervals, until there was no further significant difference between treatments (mid-April). This gave the data from which the mean values and standard errors in Table I were calculated. Subsequently, sets of two or three treatments were sampled by plots, but there were no more differences between treatments that could be called significant in terms of the variance of the last complete sampling. The results of these samplings are not given here; they appeared in Fig. 8 of the previous paper (Crowther and Richardson (2)).

As a concise summary of the meteorological conditions during the experiment, Table I contains the mean soil temperature in degrees Fahrenheit at 4 in. and the total amount of drainage in inches in the 20 in. drain gauge with bare soil. Both of these sets of data are for the interval since the previous sampling (except the first one, which is taken from the time of applying the fertilisers).

The results showed, as might have been expected, that the variation between individual values of a treatment was greater where nitrogen had recently been added than on the untreated plots or those treated some time earlier. This was particularly noticeable in the ammonia values, where a simple test confirmed that the variance was higher with high treatment means than with low ones.

Table I. *Soil inorganic nitrogen, Stackyard Field.*

Winter dressings, December 6th, 1929; spring dressing, February 17th, 1930.									
	Dec. 18, 1929	Dec. 31, 1929	Jan. 14, 1930	Jan. 28, 1930	Feb. 18, 1930	Mar. 4, 1930	Mar. 18, 1930	Apr. 1, 1930	Apr. 15, 1930
Ammonia N in mg./kg. dry soil (mean of six plots).									
Treatment									
1. No N	5.0	1.9	2.5	2.1	2.6	2.6	2.1	2.1	2.2
2. W. S/A	18.8	5.6	5.4	3.6	3.4	2.9	2.2	2.4	2.0
3. W. S/A + dicy.	11.6	5.5	6.0	3.0	2.9	3.8	2.2	2.3	2.2
4. W. Cy.	14.2	4.0	5.0	2.8	2.7	3.0	2.3	2.3	2.3
5. W. Cy. + dicy.	7.4	3.1	4.9	3.2	3.2	3.5	2.2	2.3	2.2
6. S. S/A	4.4	2.0	2.9	2.3	17.9	10.6	5.5	2.9	2.3
s.e. (on logarithmic basis) as % of treatment mean	5.7	13.5	9.5	10.7	3.8	8.6	10.4	8.9	5.6
Nitrate N.									
1. No N	1.5	1.4	1.3	1.0	1.1	1.2	0.7	1.8	0.9
2. W. S/A	3.6	2.0	1.9	1.9	1.7	1.6	0.7	1.4	0.8
3. W. S/A + dicy.	2.8	1.9	1.8	1.4	1.2	2.2	1.1	1.8	0.8
4. W. Cy.	4.2	2.4	2.5	1.9	1.4	1.7	0.8	1.5	0.9
5. W. Cy. + dicy.	3.2	2.1	1.9	1.6	1.6	1.4	0.6	1.8	1.0
6. S. S/A	2.0	1.1	1.2	0.8	1.2	2.5	1.6	1.9	0.8
s.e. (mg./kg.)	0.39	0.21	0.17	0.16	0.16	0.21	0.18	0.16	0.10
As % of treatment mean	13.7	11.6	9.7	10.7	11.6	11.8	19.6	9.6	11.5
Soil-dry matter as per cent. of fresh soil.									
General mean	78.2	79.0	78.9	78.9	79.0	79.9	79.1	81.6	79.8
Soil temperature and drainage.									
Mean temp. at 4 in. in ° F.	40.9	36.6	38.7	40.1	36.1	35.5	38.5	40.3	44.1
Drainage in inches	1.98	2.28	2.10	0.77	0.97	0.00	0.70	0.01	0.24

To allow for this, the standard errors of the ammonia values were calculated on the logarithms of the actual determinations. The values so obtained were considerably more uniform over the whole period of sampling than were the simple standard errors. For nitrates, where the range of values was much less than for ammonia, simple standard errors were calculated.

The general course of the soil ammonia and nitrate levels may be appreciated from Fig. 8 in the previous paper (Crowther and Richardson (2)). It may be added that in preparing this figure the diluting effect of soil moisture on the Carsten-Olsen extracts was overlooked, so that the values shown were too low by 8-10 per cent. This does not affect the *relative* positions of the curves. As the presentation there took no account of the treatments receiving dicyanodiamide, nor of the standard errors of the determinations, a more detailed consideration of the results follows.

The rapid disappearance of all forms of added nitrogen in both winter and early spring is noteworthy (compare Table II). The soil temperature

was, if anything, lower after the early spring than after the winter application; the initial rate of disappearance was possibly a little slower in the early spring, but in both seasons the nitrogen added as sulphate of ammonia was removed so rapidly that three-fourths had gone in 3-4 weeks. Subsequent work (Richardson(3)) has shown that later in the spring the rate of disappearance increases, so that only a few days may then be required for the removal of three-fourths of the nitrogen from a heavy dressing of sulphate of ammonia. What proportion of this nitrogen is secured by the plant is a question that will be considered later in connection with the recovery of the nitrogen in the herbage.

Table II. *Disappearance of soil inorganic (ammonia and nitrate) nitrogen.*

Differences from untreated soil, as per cent. of nitrogen added. Winter dressings applied December 6th; spring dressing February 17th.									
	Dec. 18	Dec. 31	Jan. 14	Jan. 28	Feb. 18	Mar. 4	Mar. 18	Apr. 1	Apr. 15
W. S/A	80	22	18	12	7	4	0	0	-1
W. S/A + dicy.	40	20	20	6	2	10	2	1	0
W. Cy.	60	16	18	8	2	4	1	0	0
W. Cy. + dicy.	20	10	15	8	6	6	0	1	0
S. S/A	—	—	—	—	77	46	22	4	0

The differences between treatments may best be examined for the separate ammonia and nitrate determinations, comment being restricted to statistically significant differences. With *winter sulphate of ammonia*, although the soil ammonia fell off rapidly at first, it remained above that of the untreated soil for some 10 weeks. Nitrate did not accumulate to the extent of more than 1 or 2 mg./kg., but it remained above the untreated soil for the same period. With *spring sulphate of ammonia*, although the initial rate of disappearance of the added nitrogen was about the same as in winter, disappearance was more rapid when the soil warmed up in April, so that the soil ammonia remained above the untreated for only 6 weeks. The added inorganic nitrogen was completely removed from the soil by mid-April. Nitrate accumulated even less than in the winter and disappeared still more rapidly, a significant difference from the untreated soil lasting for only 4 weeks. As there was little drainage for some time after the sulphate of ammonia was applied, the low nitrate level cannot be attributed to leaching.

Although the soil ammonia from *winter calcium cyanamide* was frequently below that from sulphate of ammonia the difference was significant on only two occasions—on the first sampling, 10 days after application, and again in mid-February. Soil nitrate, rather unexpectedly,

showed the opposite tendency, being for the first 6 weeks slightly higher with calcium cyanamide, although the difference was only once significant. This might be attributed to a reduction in nitrate uptake by the herbage following a slight check to the herbage from the cyanamide.

The ammonia from calcium cyanamide was completely removed from the soil before that from sulphate of ammonia, for the difference from the untreated soil had fallen below significance 7 weeks after application. Nitrate nitrogen persisted a little longer, but it had gone by the tenth week.

It is thus clear that, so far as soil inorganic nitrogen is concerned, the winter application of calcium cyanamide did not give a more prolonged supply of nitrogen than did sulphate of ammonia.

If calcium cyanamide had appreciably checked nitrification a higher ammonia level might have been expected from cyanamide than from sulphate of ammonia after the first few days. This was observed in uncropped soils in pots(2). Its absence here cannot, however, be taken as evidence that there was no delay in nitrification, for the extra ammonia may have been removed directly by the herbage(4).

In examining the behaviour of soils which received *dicyanodiamide*, it must be remembered that only half of the added nitrogen was present as sulphate of ammonia or calcium cyanamide. This was reflected in the soil ammonia values 10 days after application, when both treatments receiving dicyanodiamide were below those without dicyanodiamide. Subsequently, however, there were no significant differences (except once, with sulphate of ammonia in mid-February). Greater precision in examining the action of dicyanodiamide was obtained by comparing the twelve plots receiving half their nitrogen in this form with the twelve receiving all their nitrogen as sulphate of ammonia or calcium cyanamide. This confirmed the observation that there were no significant differences in soil ammonia after the first sampling; soil nitrate values were significantly lower in the presence of dicyanodiamide in three out of the first four samplings, but not subsequently. Soil nitrates were higher in the treatments receiving dicyanodiamide than in the untreated soil, in the first four samplings. Dicyanodiamide therefore retarded, but did not completely inhibit nitrification in this experiment. There was no evidence in the later measurements that dicyanodiamide nitrogen added in the winter became available in the spring.

THE GROWTH AND YIELD OF HERBAGE.

(a) *Stackyard Field experiment*, 1929-30.

The soil of this experiment has already been described, while its agricultural history appears at the head of Table III. The area chosen for the experiment was almost level; it was prepared by removing flints and dung, rolling, and mowing with a special lawn mower set to cut high and fitted with a basket (designed by R. G. Warren) to catch the herbage as completely as possible. The fertilisers were diluted with sand to uniform bulk and sprinkled on by hand from a vessel with a perforated end. The six randomised blocks of the experiment were grouped in two major blocks to give a compact lay-out.

The procedure of sampling the herbage varied at first, but in the later cuts the whole produce of each plot was weighed as soon as cut; it was then mixed and small handfuls were taken at random to make up a sample of 1 lb., which was kept in a relatively large, porous bag to avoid overheating. These samples were transferred to a warm room at intervals during the day, and, after being spread and partly dried there, were finally dried for 2 days in a steam-oven. After weighing for dry matter the samples were either ground separately, or bulked and then subsampled and ground for nitrogen determinations.

Herbage. The effect of the winter applications was slow in appearing, but by the end of January some of the no-nitrogen plots could be distinguished by their paler appearance. When spring sulphate of ammonia was applied, in mid-February, the plots with winter nitrogen were all greener than the remainder. The effect of the spring nitrogen was visible after about a month (the average temperature being low in the interval), and it was well developed by the beginning of April. By this time only a trace of the spring nitrogen remained in the soil in the inorganic form.

When the first cut was made, at the end of April, spring sulphate of ammonia had given a strikingly heavier and greener growth of grass than the winter treatments, while the no-nitrogen and some of the dicyanodiamide plots looked relatively thin and pale. Subsequent cuts were not made at regular intervals, but whenever the grass had reached a height—from 3 to 5 in.—suitable for grazing by stock under the “intensive” system.

The differences in growth with treatment were less marked with the second cut in the middle of May than they had been with the first. By the end of May, when clover was developing vigorously, it was evident

Table III. *Stackyard Field, yield and nitrogen content of herbage, 1930.*

Previous cropping. Seeds mixture sown under winter oats in April 1928. Indigenous strains used: perennial ryegrass 16 lb. per acre; cocksfoot 10 lb.; timothy 4 lb.; rough-stalked meadow grass $\frac{1}{2}$ lb.; late flowering red clover 4 lb.; wild white clover 1 lb. Grazed during 1929.

Previous manuring. 6 cwt. per acre basic slag November 27th, 1928. 8 tons per acre farmyard manure December 5th, 1928. $1\frac{1}{4}$ cwt. per acre sulphate of ammonia early autumn 1929.

Plots: 10 yd. \times 2 ft.; six replicates in six randomised blocks. All plots harrowed and rolled March 13th, 1930.

Treatments		Date Dec. 6th, 1929	No N	Treatment
No.	Symbol			
(1)	No N			
(2)	W. S/A	"	0.4 cwt. N as W. S/A	
(3)	W. S/A + dicy.	"	{ 0.2 " W. S/A	
(4)	W. Cy.	"	{ 0.2 " W. dicy.	
(5)	W. Cy. + dicy.	"	{ 0.4 " W. Cy.	
(6)	S. S/A	Feb. 18th, 1930	{ 0.2 " W. dicy.	
			0.4 " S. S/A	

Individual cuts.

Date of cutting ...	Apr. 27	May 16	June 2	July 1	Aug. 19	Nov. 13
No. of replicates ...	5	6	6	6	3	6

Yield of dry matter, cwt. per acre.

Treatment						
(1) No N	2.41	3.29	3.14	4.51	3.19	1.57
(2) W. S/A	3.31	4.42	3.19	4.64	3.53	1.80
(3) W. S/A + dicy.	2.97	4.23	3.33	5.43	3.92	1.92
(4) W. Cy.	2.86	4.12	3.33	4.84	3.58	2.06
(5) W. Cy. + dicy.	2.68	4.01	3.37	4.80	3.76	1.82
(6) S. S/A	4.79	5.70	3.24	4.06	3.55	2.05
s.e.	0.138	0.195	0.083	0.192	0.303	0.112
s.e. as % of mean	5.14	6.28	2.56	4.00	8.46	6.00

Nitrogen, as per cent. of dry matter.

(1) No N	2.49	2.50	2.63	2.49	3.01	3.32
(2) W. S/A	2.66	2.49	2.71	2.39	2.94	3.42
(3) W. S/A + dicy.	2.60	2.52	2.79	2.49	2.92	3.52
(4) W. Cy.	2.60	2.55	2.74	2.49	2.98	3.34
(5) W. Cy. + dicy.	2.59	2.51	2.72	2.43	2.82	3.40
(6) S. S/A	2.91	2.50	2.72	2.25	2.74	3.19
s.e.	0.039	0.021	—	—	—	—
s.e. as % of mean	1.48	0.84	—	—	—	—

Totals for year.

Treatment	Yield of dry matter		Yield of nitrogen	
	Cwt. per acre	As % of no N	Nitrogen, cwt. per acre	As crude protein cwt. per acre
(1) No N	18.10	100	0.485	3.03
(2) W. S/A	20.89	115	0.561	3.51
(3) W. S/A + dicy.	21.81	120	0.594	3.71
(4) W. Cy.	20.79	115	0.567	3.54
(5) W. Cy. + dicy.	20.43	113	0.546	3.41
(6) S. S/A	23.38	129	0.624	3.90
s.e.	0.604	3.34	—	—
s.e. as % of mean	2.89	—	—	—

that the clover was less vigorous on the spring sulphate of ammonia plots than elsewhere. When the third cut was taken, at the beginning of June, the direct response of the herbage to nitrogen seemed to have disappeared, while during the rest of that month the suppression of clover became more and more evident. At the time of the fourth cut, on July 1st, the spring sulphate of ammonia plots could be picked out by anyone not knowing the lay-out of the experiment, because of the relative scarcity of clover flowers, and the effect showed strikingly in photographs. The reason for this action on clover will be considered later.

The effects of the different treatments on *yield of dry matter* (Table IV) accord with these observations. All the treatments except winter calcium cyanamide + dicyanodiamide, produced significant increases over no nitrogen in the first cut, and all of them did so in the second cut. By the third cut, however, the effect of the added nitrogen had been used up (in spite of the rather heavy dressing used), for there were no significant differences on June 2nd. In the last two cuts of the year the untreated plots tended to be below the others, although the difference only reached significance with three treatments in the last cut.

Spring sulphate of ammonia was by far the most effective treatment in the first two cuts, giving yields significantly above any others. In the July cut, on the other hand, the yield was significantly below that with most of the other treatments: this may be attributed to the poorer growth of clovers.

Winter sulphate of ammonia was rather less than half as effective as the spring dressing in the first two cuts. It showed, however, no depression in yield in July. *Winter Calcium Cyanamide* was not as effective as winter sulphate of ammonia in either of the first two cuts, although the difference was significant only in the first cut. Replacing half the nitrogen by *dicyanodiamide* gave lower yields with both forms of nitrogen in both of the early cuts, but none of the differences was significant. There appeared to be a significant delayed response to dicyanodiamide with sulphate of ammonia on July 1st; this was probably due to the increased development of clovers that was observed with this treatment.

Effects on clovers. After the July cut A. R. Clapham kindly estimated the area occupied by clovers under the different treatments (by graphical plotting). He found no significant differences between no nitrogen and any of the winter treatments, although there was an increase with winter sulphate of ammonia + dicyanodiamide that was on the verge of signi-

ficance. Spring sulphate of ammonia caused a depression that was highly significant:

Treatment	...	No N	W. S/A	W. S/A + dicy.	W. Cy.	W. Cy. + dicy.	S. S/A	S.E.
Clover, % of total herbage by area		43.0	36.3	49.5	41.7	44.4	14.4	2.24

If the twelve plots in which half the nitrogen was present as dicyanodiamide are compared with the twelve plots which received a full dressing of nitrogen as winter sulphate of ammonia or calcium cyanamide, the result (without dicyanodiamide 39.0 per cent., with dicyanodiamide 46.9 per cent.) shows that there was a higher proportion of clovers with dicyanodiamide. Because of the greater number of plots involved the difference is highly significant.

These results throw some light on the reasons for clover repression with sulphate of ammonia. Possibly with a sufficiently heavy dressing, sulphate of ammonia is directly toxic to clovers, but in this experiment, although some damage to the clover foliage was observed shortly after the spring application, this disappeared. It was not until two cuts had been taken that a relative decrease in clovers became noticeable. This suggests that competition with the heavy growth of grass produced by the spring nitrogen was the chief factor in the reduction of clover.

The values for the percentage of clovers under different treatments in July show a tendency to an inverse relationship when compared with the effects of the treatments on yield in earlier cuts. Spring sulphate of ammonia gave higher yields than winter sulphate and caused a greater depression of clovers; treatments receiving dicyanodiamide gave lower yields than those without it, and higher clover percentages.

If actual treatments are neglected and the plot yields for the first two cuts (the duration of the response to nitrogen) are correlated with the percentage of clover in each plot in July, a correlation coefficient of -0.423 is obtained. Since thirty-six plots are involved in the comparison this result is highly significant ($P = < 0.01$). It shows that the greater the growth of grass in spring—the greater the competition for nutrients and light—the less was the growth of clover in summer.

Nitrogen content of herbage. For the first two cuts the percentage of nitrogen was determined on samples from individual plots. The standard errors were so low in comparison with those for yields, that subsequent determinations were made on a single bulked sample for each treatment.

The percentage nitrogen content in the first cut was greatly increased by spring sulphate of ammonia, and to a less extent, though still significantly, by winter sulphate. The effect of the other treatments was only

on the verge of significance. In the second cut there were no significant differences, showing that, although some extra nitrogen was still present in the herbage, it was no more than could be efficiently utilised by the plant in producing dry matter. In later cuts the effect of clover repression is to be seen in the relatively low nitrogen values with spring sulphate of ammonia.

The apparent *recovery of added nitrogen* from the different treatments is complicated by the clover effect and the falling off in the yield of the no-nitrogen plots in autumn. Since, however, the direct action of the nitrogen on the herbage was exhausted in the first two cuts (when there was relatively little clover in the herbage), and the effect on the clover was most marked in the later cuts, the nitrogen taken up in the first two cuts may be used to measure the "recovery." This is done in Table IV, the corresponding figures for the whole year being given also for comparison.

Table IV. *Recovery of added nitrogen. Stackyard Field, 1930.*

Treatment	Recovery in first two cuts		Recovery over whole year	
	Cwt./acre	%	Cwt./acre	%
W. S/A	0.060	15	0.076	19
W. S/A + dicy.	0.045	11	0.109	27
W. Cy.	0.035	9	0.082	23
W. Cy. + dicy.	0.027	7	0.061	15
S. S/A	0.144	36	0.139	35
S.E.	0.0113	2.8	—	—

Because the nitrogen contents had been determined on separate plot samples for the first two cuts, the standard errors of the values for nitrogen taken up, and hence for nitrogen recovery, could be calculated. The corresponding data were not available over the whole year.

It will be seen that the recovery from spring sulphate of ammonia was significantly higher than that from any of the winter treatments, while none of the differences between the winter treatments was significant. There was no indication in the recovery from the first two cuts that calcium cyanamide or dicyanodiamide had enabled the herbage to secure more nitrogen in competition with winter leaching.

The "recoveries" for the whole year were somewhat higher, with the winter treatments, than those for the first two cuts. The significance of the difference cannot be estimated, and emphasis need not be placed on it. Spring sulphate of ammonia did not show it, while it was particularly marked with winter sulphate of ammonia and dicyanodiamide, so

the clover effect may have been partly responsible. Since there was also a tendency, rarely reaching significance, for the no-nitrogen plots to give poorer yields than the others in the autumn, it is possible that a little of the added nitrogen locked up by micro-organic action in winter and spring became available in the autumn.

(b) *Stackyard Field experiment, 1930-1.*

After the last cut in 1930 sheep were allowed to graze the plots (in common with the rest of the field) for a month. The experiment was then repeated on the same plots, chiefly in order to examine under slightly different conditions the action of dicyanodiamide. In the second year this compound was applied as a dressing additional to a full application of nitrogen in the form of sulphate of ammonia or calcium cyanamide.

Because of snow and cold in early spring, the spring sulphate of ammonia was applied nearly a month later than in 1930.

The conditions were increasingly divergent from those of normal pasture, so the results from this year (Table V) will be considered in less detail than those of the previous year.

In the second year of the experiment the growth of clovers was remarkably abundant even at the time of the first cut, probably as a result, at least in part, of the repeated mowing of the previous year. The season was a late one, with a cold March, and this may also have checked the early growth of the grasses and given clovers an advantage. The clovers were fairly plentiful on the spring sulphate of ammonia plots (they had not been killed out), but they were far more dense on the others, especially on the no-nitrogen plots. The results must be examined with this in mind.

There was a significant response in yield to all forms of nitrogen in the first cut, but no significant responses in the second, at the beginning of June.

Spring sulphate of ammonia was not significantly above winter sulphate (alone or with dicyanodiamide added) in the yield of the first cut. In the third cut (early July) it was significantly below all other treatments: a more marked expression of the effect on clover observed in July of the previous year. The percentage nitrogen content of the herbage was below the no-nitrogen plots with spring sulphate of ammonia until the autumn, which must be attributed to the preponderance of clovers in the herbage of the untreated plots.

Table V. *Stackyard Field, yield and nitrogen content of herbage, 1931.*

Previous cropping, etc., see Table III. Grazed in late autumn of 1930. Same plots as in 1930.

Treatments. Nitrogen as sulphate of ammonia or calcium cyanamide, 0.4 cwt. per acre. Nitrogen as dicyanodiamide, Nos. 3 and 5, 0.2 cwt. per acre extra. Numbers and symbols (below) as in Table III.

Winter treatments applied December 10th, 1930.

Spring sulphate of ammonia applied March 13th, 1931.

Individual cuts.

Date of cutting	...	May 9	June 1	July 3	Aug. 7	Sept. 28	Dec. 11
Yield, dry matter, cwt. per acre.							

Treatment							
(1) No N	5.76	10.49	9.96	7.54	8.44	1.90	
(2) W. S/A	7.39	10.93	10.03	7.99	8.57	2.06	
(3) W. S/A + dicy.	7.84	10.85	9.67	7.52	8.95	2.24	
(4) W. Cy.	6.90	9.92	10.07	7.64	7.99	2.00	
(5) W. Cy. + dicy.	6.60	10.02	9.67	7.34	8.43	2.01	
(6) S. S/A	7.97	10.16	8.60	8.23	7.92	1.66	
S.E.	0.264	0.167	0.226	0.235	0.348	0.104	
S.E. as % of mean	3.73	1.60	2.33	3.05	4.15	5.34	

Nitrogen, as per cent. of dry matter.

(1) No N	4.14	4.08	4.01	4.00	3.68	3.50
(2) W. S/A	3.88	3.76	4.01	4.09	3.50	3.57
(3) W. S/A + dicy.	3.95	3.68	3.96	3.82	3.69	3.54
(4) W. Cy.	3.72	3.79	3.89	4.06	3.50	3.62
(5) W. Cy. + dicy.	3.68	3.74	3.88	3.83	3.56	3.55
(6) S. S/A	3.35	3.33	3.80	3.93	3.78	3.49

Totals for year.

Treatment	Yield of dry matter		Yield of nitrogen	
	Cwt. per acre	As % of no N	Nitrogen cwt. per acre	As crude protein cwt. per acre
(1) No N	44.10	100	1.745	10.9
(2) W. S/A	46.97	106	1.800	11.2
(3) W. S/A + dicy.	47.07	107	1.789	11.2
(4) W. Cy.	44.53	101	1.687	10.5
(5) W. Cy. + dicy.	44.08	100	1.646	10.3
(6) S. S/A	44.54	101	1.613	10.1
S.E.	0.815	—	—	—
S.E. as % of mean	1.80	—	—	—

Winter sulphate of ammonia and winter calcium cyanamide showed greater differences in yield than in the previous season; if the twelve plots receiving each of these treatments (ignoring additions of dicyanodiamide) are compared, sulphate of ammonia was significantly better than calcium cyanamide in both of the first two cuts.

Adding dicyanodiamide to a full winter dressing of sulphate of am-

monia or calcium cyanamide had no significant effect on the yield in any of the cuts. Taking the two seasons together, dicyanodiamide would seem to have been inert so far as its effect on yield of grass was concerned; it may possibly have been washed out of the soil by winter rains. Its formation when superphosphate is mixed with calcium cyanamide offers no advantage for the manuring of grassland.

In the last cut of 1931, as in 1930, the no-nitrogen plots gave a lower yield than any of the winter nitrogen plots, but the difference was only once significant. The yields in the last cut in both years were small, and if this result indicates that some of the winter nitrogen was being released late in the following autumn the effect was nevertheless almost negligible.

(c) *Sawyers Field experiment, 1930-1.*

The object of this experiment was to make a direct comparison between calcium cyanamide and sulphate of ammonia in both autumn and spring. The field was similar in its history to Stackyard Field (see Table VI), and was grazed—latterly by pigs and sheep—up to the beginning of the experiment. Plots were prepared and mown, and treatments were applied in the manner already described. Although rain fell soon after the autumn dressings were applied, the calcium cyanamide plots suffered damage which remained evident throughout the winter and probably influenced the yields obtained.

Because a cold spell was followed by drought, the spring dressings were withheld until rain came, which was not until the beginning of April. By this date the autumn sulphate of ammonia plots and to a less extent the calcium cyanamide ones were distinctly greener than the others. That is to say, there were signs of an "early bite" from the autumn nitrogen in a season when early application of spring nitrogen offered difficulties.

It was intended to alternate mowing and grazing in this experiment, in order to approximate more nearly to pasture conditions, but only one cut was actually taken. In consequence there was no opportunity to examine residual effects, but the results of the Stackyard experiment conducted at the same time suggest that the direct effects of the added nitrogen were largely measured in the first cut.

The results showed a significant response in yield to all treatments except autumn calcium cyanamide, while there was no significant difference between the yields with autumn and spring sulphate of ammonia, or between spring sulphate of ammonia and spring calcium

cyanamide. Both spring treatments raised the percentage nitrogen content significantly, but the autumn dressings did not. In other words, the nitrogen taken up in the autumn was fully utilised by the plant in building up further dry matter.

Table VI. *Sawyers Field, yield and nitrogen content of herbage, 1931.*

Previous cropping. Seeds mixture sown under barley in April 1928. Indigenous strains used: 10 lb. perennial ryegrass, 8 lb. cocksfoot, 2 lb. timothy, 2 lb. meadow fescue, 2 lb. rough-stalked meadow grass, 1 lb. early-flowering and 2 lb. late-flowering red clover, 1 lb. wild white clover, 2 lb. chicory. Grazed during 1929 and 1930.

Previous manuring. $1\frac{1}{2}$ cwt./acre sulphate of ammonia, autumn 1929; 1 cwt./acre sulphate of ammonia, spring 1930.

Basal dressing. Superphosphate = 0.4 cwt. P_2O_5 /acre; sulphate of potash = 0.4 cwt. K_2O /acre; the potash was applied to only one-half of each plot.

Plots: 10 yd. \times 1 yd.; five replicates in five randomised blocks.

Treatments: (1) No nitrogen.

(2) Autumn sulphate of ammonia, 0.4 cwt. N/acre, November 7th, 1930.

(3) Autumn calcium cyanamide, 0.4 cwt. N/acre, November 7th, 1930.

(4) Spring sulphate of ammonia, 0.4 cwt. N/acre, April 2nd, 1931.

(5) Spring calcium cyanamide, 0.4 cwt. N/acre, April 2nd, 1931.

Cut. April 30th, 1931.

Treatment	Yield dry matter cwt./acre	Nitrogen % in dry matter	Nitrogen in herbage cwt./acre	Recovery of nitrogen	
				cwt./acre	%
(1) No N	10.0	3.60	0.360	—	—
(2) A. S/A	13.2	3.60	0.475	0.115	29
(3) A. Cy.	10.4	3.56	0.370	0.010	2
(4) S. S/A	13.0	3.96	0.515	0.150	38
(5) S. Cy.	12.2	3.83	0.467	0.107	27
s.e. actual	0.34	0.052	—	—	—
s.e. as % of general mean	2.8	1.4	—	—	—

The plots were halved for potash, but there was no significant response to potash, either over the whole experiment or in interaction with the nitrogen treatments.

The recovery of nitrogen from the different treatments showed that the autumn dressings were less efficient than the spring ones, although with sulphate of ammonia the difference was not great. The recovery from spring calcium cyanamide was less than from sulphate of ammonia, but again the difference was not very large; in view of the short period—4 weeks—between application and cutting this may be taken as indicating quite rapid availability of the cyanamide nitrogen.

DISCUSSION.

The lawn-mowing technique. A full examination of the advantages and disadvantages of this method of studying grassland problems would require more space than is available here. Obviously it will tend to cause changes in the mixed herbage different from those ensuing under controlled grazing. At the same time it keeps the herbage in the immature condition, and to this extent it approximates more nearly to intensive grazing than to meadow conditions.

The method of repeated mowing represents, indeed, an intermediate stage between pasture and meadow. Whereas the herbage is maintained in the immature state, the plant nutrients it contains are completely removed from the land instead of being partly returned. The most important bearing of this on the present work is that under grazing the return of much of the nitrogen in excreta would have prolonged the effect of the manures beyond that observed with mowing.

Since the experiments were laid out on pasture fields which were grazed until shortly before the work began, the first cut or two taken with the mower may fairly be regarded as representative of pasture conditions.

For exploring the behaviour of nitrogenous fertilisers the lawn-mowing technique offers definite advantages. By its means a replicated experiment is readily carried out, and considerable accuracy is obtained (as may be seen in the low standard errors of yields in the present experiments). Measures of the duration of the direct effect of the nitrogen, and of the nitrogen recovery in the early cuts, are possible precisely because there are no animals to complicate the results. Further, if the direct recovery of added nitrogen in the herbage is worse from one treatment than from another, grazing stock may lengthen the period over which the nitrogen acts, but they can hardly increase the relative recovery from the poorer treatment.

Results obtained by this technique are directly applicable to the production of grass for artificial drying, by repeated mowing, a branch of agriculture which is now being developed in some parts of the country.

The behaviour of nitrogen in grassland soils. Conclusions from this and some more recent work have been briefly reviewed elsewhere (Richardson (5)). It will be sufficient to point out here that the ammonia level tends to be higher, relative to the nitrate level, in grassland soils than in arable ones. A similar result has been recorded by Eggleton, Lewis and Page(6) and by Penman(7). This higher ammonia level is

probably attributable to less vigorous nitrifying activity in the undisturbed soil of grassland, since the extra ammonia disappears from many of the soils when they are allowed to nitrify under optimum conditions in the laboratory.

Other grassland soils nitrify poorly even in the laboratory, especially if their reaction is much on the acid side. In some of these it has been shown (Richardson (4)) that although added ammonia disappears rapidly when the grass is growing it remains in the soil if the herbage is removed. Clearly, as Hall, Miller and Gimingham (8) suggested many years ago, grass can take up its nitrogen directly as ammonia without the need for preliminary nitrification. Even in non-acid grassland soils, since nitrification is relatively less vigorous than in arable soils, it is likely that much of the nitrogen added in a dressing of an ammonium salt is taken up directly in this form, although a part may also be absorbed as nitrate.

In the present experiments the relatively rapid disappearance of ammonia and very slight production of nitrate in both winter and early spring suggested that ammonia was being taken up directly. However, the greater part of the nitrogen added did not appear in the herbage at all (less than 40 per cent. was recovered in any experiment). While there may have been some loss in the winter from leaching (which was shown—Crowther and Richardson (2))—to cause a fairly rapid loss of winter nitrogen from arable soil), this would not account for the loss when the grass was beginning to grow rapidly in the spring. It seems most probable that the poor recovery of added nitrogen was due to competition between the grass roots and those micro-organisms engaged in breaking down carbohydrates and building up nitrogenous organic matter in the soil.

It is common knowledge that old grassland soils are much richer in nitrogenous organic matter than are corresponding arable soils: this nitrogen becomes available when the soils are ploughed up. Presumably the loss of nitrogen in these experiments represents the other side of the picture: a "locking up" of nitrogen in young grassland soils. Later cuts showed that a little of this nitrogen became available in the autumn, but most of it remained in the soil.

Management and protein production. On this young grassland the conversion of added nitrogen into protein was rather inefficient. Although enough nitrogen was added to give 2.5 cwt. per acre of crude protein, during the first season on Stackyard Field only 0.9 cwt. per acre of additional crude protein was produced from the spring sulphate of ammonia dressing. This low figure was not entirely due to clover sup-

pression, since similarly low recoveries of nitrogen were obtained in the early cuts on both Stackyard and Sawyers Fields.

The effect of the suppression of clovers was more marked in the second year of the Stackyard experiment. When the spring sulphate of ammonia treatment was repeated the total production of crude protein in the second season was actually less by 0.8 cwt. per acre than on the plots without nitrogen. In both years, also, the yields were depressed in July by the spring sulphate of ammonia.

In ordinary farming some suppression of clovers is likely to occur after heavy spring dressings of nitrogen, and in estimating the practical value of these dressings due attention should be paid to the probable depression in yield of herbage and especially of clovers and protein during the summer. Under some conditions of farming the "early bite" may be so important that summer keep may with advantage be sacrificed.

With careful management, however, the depression of clovers may be made much less than it was in these experiments. Here the clover undoubtedly suffered from competition with the vigorously growing grasses, during the rather long intervals between cuts. This might have been reduced by more frequent cutting on the manured than on the unmanured plots. Martin Jones has demonstrated that, by suitable control of the grazing and resting of pastures, either grassy or clovery swards may be produced almost at will. Further, he has shown⁽⁹⁾ that with suitable management relatively heavy manuring with nitrogen need not disturb the balance between the competing species.

The influence of different nitrogenous manures on yield of herbage. Bringing together the results of the three experiments, spring applications of nitrogen always secured a good immediate response, but it lasted for only one or two cuts. In the spring calcium cyanamide was practically as effective as sulphate of ammonia.

Winter (December) applications gave a measurable response, but much less than the spring dressings. Over the whole year they appeared to more advantage because they caused less suppression of clovers.

Late autumn (November) sulphate of ammonia proved better than the December applications and almost as good as a spring one, probably because the grass was not yet dormant and secured enough nitrogen to give it a good start in spring. Calcium cyanamide applied at this time caused more injury than in December (when the grass was dormant) or in spring (when vigorous fresh growth soon repaired any injury that occurred).

Concerning *calcium cyanamide*, and *dicyanodiamide*, these experiments have not shown any advantage attributable to the supposed slow availability of the nitrogen. Under favourable spring conditions, as the Sawyers' experiment showed, cyanamide nitrogen is nearly as rapidly available as that of sulphate of ammonia. When cyanamide nitrogen is applied in late autumn or winter it checks the herbage, and puts the plant at a disadvantage in its competition with leaching or soil micro-organisms; the recovery of the herbage in spring comes too late for the best use to be made of the nitrogen.

SUMMARY.

1. Ammonia added as sulphate of ammonia disappeared rapidly from a pasture grassland soil, while very little nitrate accumulated. In winter or early spring three-fourths of the added nitrogen had gone in less than 4 weeks. After the first fortnight there was little difference in the soil inorganic nitrogen from calcium cyanamide and from sulphate of ammonia. A moderate dressing of dicyanodiamide slightly reduced but did not inhibit nitrification; it did not appreciably retard the disappearance of inorganic nitrogen from the soil in winter.

2. Winter applications of sulphate of ammonia produced less increase in yield or nitrogen content of repeatedly mown herbage than did spring ones. A late autumn application was almost as effective as a spring one. Calcium cyanamide in late autumn or early winter was on the whole less effective than sulphate of ammonia, but in spring the two were substantially equal. There was little evidence that calcium cyanamide was "slow acting" in comparison with sulphate of ammonia. Dicyanodiamide was practically inert so far as the effect of winter dressings on yield or nitrogen uptake was concerned.

3. Under repeated mowing the response of the herbage to a 2 cwt. dressing of sulphate of ammonia (or other nitrogenous fertiliser) was rapidly exhausted. Later in the year there was a reduction in yield with spring sulphate of ammonia, resulting from a depression of clovers in summer through competition with the heavier growth of grass in the spring.

4. The recovery of added nitrogen in the herbage was, at best, less than 40 per cent. This may have been due in part to its locking up by microbiological action in the soil organic matter.

ACKNOWLEDGMENTS.

The author's thanks are due to Dr E. M. Crowther for his helpful advice in the preparation of this paper, and to other members of the staff of the Chemistry Department for their assistance in harvesting and analysing the herbage in these experiments.

REFERENCES.

- (1) RICHARDSON, H. L. *J. Agric. Sci.* (1932), **22**, 348.
- (2) CROWTHER, E. M. and RICHARDSON, H. L. *J. Agric. Sci.* (1932), **22**, 300.
- (3) RICHARDSON, H. L. *Nature* (1932), **130**, 96.
- (4) ——— "The relationship between soil reaction and the absorption of ammonia and nitrate by grasses." Paper read before Commission IV, International Society of Soil Science, Copenhagen, 1933. Unpublished.
- (5) ——— *Agric. Progress* (1933), **10**, 160.
- (6) EGGLETON, W. G., LEWIS, A. H. and PAGE, H. J. *Chem. and Ind.* (1932), **51**, 537.
- (6 a) ——— *J. Agric. Sci.* (1934), **24**, 416.
- (7) PENMAN, F. *J. Agric. Victoria* (1931), **29**, 439.
- (8) HALL, A. D., MILLER, N. H. J. and GIMMINGHAM, C. T. *Proc. Roy. Soc. B* (1908), **80**, 196.
- (9) JONES, MARTIN G. *Emp. J. Exp. Agric.* (1933), **1**, 122.

(Received May 18th, 1934.)

[*Reprinted from the Journal of Agricultural Science*, Vol. XXV. Part I.]

[*All rights reserved.*]

STUDIES ON CALCIUM CYANAMIDE¹.

V. THE UTILISATION OF CALCIUM CYANAMIDE IN POT CULTURE EXPERIMENTS.

By H. L. RICHARDSON AND E. M. CROWTHER.

*(Chemistry Department, Rothamsted Experimental Station,
Harpenden, Herts.)*

(With Three Text-figures.)

IN the first paper of this series⁽¹⁾ we discussed the chemical aspects of investigations continued for many years in this laboratory on the fertiliser value of "Calcium Cyanamide" (the commercial form of calcium cyanamide, CaCN_2). We showed that, contrary to some of the earlier views, the initial stages of its decomposition in the soil, through urea to ammonia, proceed very rapidly and are complete long before the nitrification of the ammonia in ammonium salts added at the same time. The final stage—the production of nitrate—may suffer a delay which is often greater than the time required for the initial decomposition of the cyanamide. This delay was shown by B. K. Mukerji⁽²⁾ to be due to the toxic action of the cyanamide nitrogen on the nitrifying organisms. The evidence on which these conclusions were based was derived from experiments in flasks and in uncropped pots in the greenhouse, from the early stages of pot experiments with barley, before the plant was large enough to cause disturbance, and, finally, from field experiments.

In the present paper we present the results of further soil analyses conducted throughout the growth of barley and mustard plants, in pot cultures, and endeavour to relate the differences in growth of the crops to the course of the decomposition of the added fertilisers. Pot experiments are particularly suited to such investigations, for they make it possible to compare several soils, under uniform though admittedly artificial conditions, without disturbance from unknown leaching losses. Since, however, the pot culture conditions are so artificial it is in the highest degree unsafe to transfer the results directly to practice.

¹ Several past and present members of the Rothamsted staff collaborated in the work described in this series of papers and, although it proved impossible to publish the contributions separately, the names of those responsible for the major experiments are given in the text, tables or figures.

Pot experiments provide information which may be used in interpreting the results of field experiments, but actual trials in the field are necessary to decide the practical value of a fertiliser. The results of field experiments on the use of calcium cyanamide as a fertiliser and in weed control, as well as on its effect on germination, are described separately in the *Empire Journal of Experimental Agriculture* (3).

PRELIMINARY EXPERIMENTS IN POTS.

Series of pot cultures comparing ammonium sulphate and calcium cyanamide on barley and mustard were conducted by R. B. Dawson (4) in 1927 and by the late A. J. Walker in 1927. The soils used in these experiments are described on pp. 135-9 together with those for the main experiments. The conditions in these preliminary experiments proved unsuitable for an adequate test, but the results are quoted to illustrate the effects of heavy additions of fertilisers on barley sown too late to allow the nitrogen absorbed to be utilised efficiently. The barley ears formed poorly, the grain/straw ratio was unduly low, and the nitrogen content of the grain was extremely high.

In 1926 it was noted that the plants with calcium cyanamide in the Rothamsted soil tillered more and had more deeply coloured leaves than those with ammonium sulphate. In the Millstone Grit soil at an early stage there were more tillers from calcium cyanamide, but the advantage was soon lost.

The final yields summarised in Table I show a slight advantage of calcium cyanamide over ammonium sulphate in the Rothamsted but not

Table I. *Pot experiments on barley (late sown) 1926—
R. B. Dawson's data.*

Comparison of ammonium sulphate (S) and calcium cyanamide (Cy.), each supplying 0.61 gm. nitrogen per pot of 11.5 kg. soil. Manures added and barley sown April 21; harvested August 23. Soils: Millstone Grit (M.G.) and Rothamsted Hoos Field (R.H.). All data in grams per pot and means of four pots.

Fertiliser	Soil ...			Rothamsted Hoos		
	No N	S	Cy.	No N	S	Cy.
Dry grain	10.4	17.8	17.3	6.5	16.9	19.3
Dry grain + straw	38.5	58.1	56.3	22.5	52.4	56.2
Nitrogen in grain	0.244	0.477	0.485	0.144	0.325	0.360
Nitrogen in grain + straw	0.379	0.724	0.730	0.221	0.487	0.526

in the Millstone Grit soil. In a mustard experiment with five soils in the autumn of 1926 (Table II) calcium cyanamide gave poorer results than ammonium sulphate in the Millstone Grit soil and in a Fen soil; the two fertilisers were substantially equal in the other three soils. It was noted

that on the two acid soils (W.S. and M.G.) ammonium sulphate caused more browning and withering of the leaf margins than calcium cyanamide, probably through a slightly greater acidification of the soil.

Table II. *Pot experiments on mustard 1926—R. B. Dawson's data.*

Comparisons of ammonium sulphate (S) and calcium cyanamide (Cy.), each supplying 1.22 gm. nitrogen per pot of 23 kg. soil. Manures added and mustard sown September 6; harvested November 25. Soils: Millstone Grit (M.G.), Woburn (W.S.), Leagrave (L.C.), Rothamsted Hoos Field (R.H.) and Fen. All data in grams per pot and means of four pots.

Soil ...	M.G.	W.S.	L.C.	R.H.	Fen
Dry crop					
No N	43.3	34.4	38.7	25.6	38.5
S	49.4	37.0	43.9	39.0	51.0
Cy.	39.9	37.0	46.6	37.8	42.1
Nitrogen in crop					
No N	1.67	1.46	1.13	0.64	1.32
S	2.16	1.83	1.99	1.66	2.02
Cy.	1.97	1.85	1.95	1.58	1.66

Table III. *Pot experiments on barley (late sown) 1927—the late A. J. Walker's data.*

Comparisons of single and double dressings of ammonium sulphate (S) and calcium cyanamide (Cy.) in pots with 11 kg. soil. Manures added and barley sown March 28–30. In Millstone Grit (M.G.) and Woburn (W.S.) soils, harvested August 13. In Rothamsted Hoos Field (R.H.), Leagrave (L.C.) and Fen soils germination was faulty and barley was resown on April 25 and harvested on September 1. All data in grams per pot and means of four pots.

Soil ...	M.G.	W.S.	L.C. (resown)	R.H. (resown)	Fen (resown)
Dry grain					
No N	12.4	14.6	8.5	5.1	14.0
0.32 N as S	15.2	15.2	12.2	11.2	13.1
0.32 N as Cy.	13.8	17.4	12.0	12.4	13.7
0.64 N as S	16.6	10.9	11.5	9.3	11.3
0.64 N as Cy.	13.1	13.2	10.6	9.9	12.2
Dry grain + straw					
No N	28.2	30.8	20.4	12.7	35.0
0.32 N as S	36.3	36.1	29.9	31.0	34.7
0.32 N as Cy.	35.8	36.7	29.1	31.8	36.5
0.64 N as S	39.2	32.1	29.7	26.6	33.8
0.64 N as Cy.	34.5	34.1	27.3	27.9	34.7
Nitrogen in grain					
No N	0.146	0.188	0.108	0.069	0.206
0.32 N as S	0.307	0.277	0.240	0.192	0.327
0.32 N as Cy.	0.289	0.294	0.246	0.199	0.316
0.64 N as S	0.424	0.302	0.343	0.261	0.366
0.64 N as Cy.	0.330	0.313	0.306	0.369	0.357
Nitrogen in grain + straw					
No N	0.221	0.240	0.148	0.092	0.311
0.32 N as S	0.469	0.391	0.338	0.314	0.503
0.32 N as Cy.	0.476	0.392	0.343	0.376	0.495
0.64 N as S	0.641	0.526	0.512	0.412	0.628
0.64 N as Cy.	0.558	0.498	0.451	0.406	0.608

In a small experiment on mustard on Rothamsted soil, with a very heavy rate of application (1 gm. of nitrogen per pot of 11 kg. soil), R. B. Dawson found that the substitution of half of the nitrogen in calcium cyanamide by dicyanodiamide greatly reduced the yield and halved the nitrogen recovery. Dicyanodiamide equivalent to the added calcium cyanamide or ammonium sulphate reduced the crop to one-sixth and allowed practically no nitrogen recovery in excess of the unmanured pots. The familiar symptoms of dicyanodiamide poisoning—whitening of the leaf margins—were well shown and persisted throughout growth.

The 1927 series (Table III) was set up parallel with the uncropped pots described in the first paper⁽¹⁾ of this series, but, owing to faulty germination, it was necessary to resow three of the soils at a later date. Plant development was poor throughout, and many of the ears failed to emerge before harvest. In four of the five soils the double rate of application of ammonium sulphate gave lower yields than the single rate, and in two of them even lower yields than the unmanured. The double rate of calcium cyanamide gave lower yields than the single rate in all soils, but the differences between calcium cyanamide and ammonium sulphate were small and inconsistent, except in the Millstone Grit soil. The experiment was concerned rather with the harmful effects of excess nitrogen on a late crop (possibly with additional disturbance from dicyanodiamide) than with the normal utilisation of the fertilisers.

POT EXPERIMENTS, 1928–31.

The fact that calcium cyanamide twice gave poor results in the Millstone Grit soil, which was known to nitrify slowly and to exhibit a marked retardation of nitrification with calcium cyanamide, led to its more thorough examination. In 1928, 1929, 1930 and 1931, W. E. Brenchley conducted pot experiments on barley and mustard in the Millstone Grit and other soils, using a uniform technique with early sown barley, and, generally, with much lower rates of application of the nitrogenous fertilisers than in the preliminary experiments. In order to explore a wide range of concentrations of the fertilisers, several rates of application were compared in three of the years.

The soils chosen for this work had widely differing characteristics, as may be seen from their responses to nitrogen and from the mechanical analyses in Table IV. The soil reactions at the end of the experiments have already been given in the first paper of the series (1), p. 330).

The Fen soil, from Cambridgeshire, was of the *Niederungsmoor* type. It was rich in calcium carbonate as well as in organic matter, and gave

so much readily available nitrogen that an extra dressing of nitrogen had little, if any, effect. In consequence, it was not used in the main series.

Table IV. *Mechanical analyses of soils used in pot experiments (on oven-dry basis).*

Soil ...	Fen (1926-7)	M.G.	L.C.	R.B.	W.S.
Coarse sand	19.8	24.4	7.6	7.9	57.8
Fine sand	15.9	32.6	17.3	43.2	24.4
Silt	5.4	16.6	9.4	26.8	5.5
Clay	12.5	18.4	20.8	17.8	6.8
Carbonates	9.1	0.0	33.2	0.1	0.0
Air-dry moisture	20.1	3.2	5.2	3.2	1.6
Loss by solution	3.2	1.2	1.7	0.9	0.7
Difference	+ 14.0	+ 3.6	+ 4.8	+ 0.1	+ 3.2
Total	100.0	100.0	100.0	100.0	100.0
Loss on ignition	16.4	6.6	6.9	5.6	3.3

The Millstone Grit soil (M.G.) was an acid loam from the Pennines, near Stalybridge, Cheshire; in its natural state it was too acid for barley. Accordingly, it received additions of calcium carbonate sufficient to raise its pH to about 6.5. The soil had, when limed, considerable reserves of decomposable organic matter which slowly yielded ammonia and nitrate during the growth of the crop; nitrification was begun more slowly than in the other soils. The response to added nitrogen was generally low, and the barley grain produced was rich in nitrogen.

The Leagrave calcareous soil (L.C.), which came from arable land at the foot of the Chiltern chalk escarpment, is to be classified as a *rendzina*. It also proved to be rich in readily available nitrogen, and it nitrified rapidly.

The Rothamsted soil (R.H.) used in 1928 and 1929 was a heavy loam, taken from an area in Hoos Field which had been deliberately impoverished by continued arable cropping without manure. It still contained enough calcium carbonate from applications early last century to be alkaline. It gave low nitrate levels and a marked nitrogen response. The other Rothamsted soil (R.B.) used in 1929 and 1931 came from Broadbalk Field, from a strip adjoining the continuous wheat plots but markedly different from them in composition and behaviour. Until recent years its site was a part of the wide grass headland—the actual “Broad Balk”—which formerly surrounded the wheat field, after the old Hertfordshire custom described by William Cobbett. It was slightly acid and had no reserves of calcium carbonate, but it had considerable reserves of readily available nitrogen. The two Rothamsted soils were

Table V. *Pot experiments on barley and mustard 1928, 1929, 1931*
(conducted by W. E. Brenchley).

Comparisons of increasing amounts of ammonium sulphate (S) and calcium cyanamide (Cy.) in pots with 10 kg. soil. Soils: Millstone Grit (M.G.), Leagrave (L.C.), Rothamsted Hoos Field (R.H.), Rothamsted Broadbalk (R.B.) and Woburn (W.S.). All data in grams per pot and means of four pots, in 1928 and 1931, and of six pots in 1929.

A. Barley, 1928 (sown March 9, harvested August 9–11).

Soil ...	M.G.		L.C.		R.H.		W.S.	
Fertiliser ...	S	Cy.	S	Cy.	S	Cy.	S	Cy.
Dry grain								
No N	18.9	—	18.7	—	13.6	—	12.6	—
0.106 N	22.7	22.3	21.5	22.7	17.8	17.4	16.1	17.5
0.212 N	23.8	24.4	23.0	24.4	20.9	19.4	19.9	19.0
0.424 N	23.9	25.7	25.0	25.5	25.5	24.7	26.3	24.3
Dry grain + straw								
No N	42.6	—	42.5	—	30.4	—	27.8	—
0.106 N	49.1	50.6	48.2	49.5	40.2	39.2	36.3	39.8
0.212 N	51.5	53.1	50.5	52.2	47.6	45.2	45.3	43.3
0.424 N	51.6	57.0	53.3	55.1	55.2	54.3	59.2	54.2
Nitrogen in grain								
No N	0.357	—	0.272	—	0.220	—	0.226	—
0.106 N	0.412	0.417	0.308	0.340	0.245	0.251	0.252	0.271
0.212 N	0.457	0.459	0.356	0.385	0.282	0.272	0.298	0.300
0.504 N	0.504	0.576	0.430	0.441	0.398	0.359	0.393	0.382
Nitrogen in grain + straw								
No N	0.446	—	0.345	—	0.277	—	0.276	—
0.106 N	0.517	0.525	0.413	0.448	0.304	0.311	0.315	0.339
0.212 N	0.576	0.592	0.459	0.499	0.355	0.340	0.371	0.371
0.504 N	0.668	0.763	0.567	0.586	0.498	0.453	0.500	0.482

B. Mustard, 1928 (sown August 28, harvested December 10).

Dry crop								
No N	12.0	—	7.0	—	3.2	—	10.4	—
0.106 N	13.0	14.8	9.4	9.6	4.4	5.8	13.6	15.4
0.212 N	15.7	14.9	12.0	12.2	7.4	6.3	16.6	17.0
0.504 N	18.0	18.4	16.9	16.4	11.5	9.4	21.7	20.1
Nitrogen in crop								
No N	0.417	—	0.222	—	0.077	—	0.277	—
0.106 N	0.428	0.456	0.266	0.271	0.105	0.124	0.343	0.392
0.212 N	0.523	0.505	0.315	0.322	0.162	0.158	0.468	0.463
0.504 N	0.589	0.664	0.454	0.410	0.279	0.248	0.597	0.566

C. Barley, 1929 (sown March 18, harvested August 15–16).

(In the series given in brackets the whole of the fertiliser was added in the top quarter of the pot.)

Soil ...	M.G.		R.H.		R.B.	
Fertiliser ...	S	Cy.	S	Cy.	S	Cy.
Dry grain						
No N	27.6	—	5.7	—	12.8	—
0.214 N	28.6	28.3	12.4	13.4	20.8	21.1
(0.214 top) N	(28.5)	(20.1)	—	—	(20.9)	(20.6)
0.424 N	27.7	28.5	22.0	21.1	25.2	25.2

Table V (*cont.*)

Soil	M.G.		R.H.		R.B.	
Fertiliser ...	S	Cy.	S	Cy.	S	Cy.
Dry grain + straw						
No N	59.7	—	13.0	—	29.8	—
0.214 N	62.0	61.3	27.9	30.0	47.2	47.5
(0.214 top) N	(61.1)	(62.5)	—	—	(47.2)	(46.2)
0.424 N	60.6	62.1	47.4	45.4	56.3	56.3
Nitrogen in grain						
No N	0.511	—	0.075	—	0.188	—
0.214 N	0.605	0.591	0.145	0.162	0.264	0.272
(0.214 top) N	(0.596)	(0.628)	—	—	(0.266)	(0.253)
0.424 N	0.643	0.686	0.283	0.269	0.379	0.377
Nitrogen in grain + straw						
No N	0.676	—	0.095	—	0.239	—
0.214 N	0.788	0.779	0.190	0.208	0.346	0.354
(0.214 top) N	(0.774)	(0.818)	—	—	(0.350)	(0.329)
0.424 N	(0.846)	0.909	0.369	0.342	0.512	0.495

D. Barley, 1931 (sown March 4-6, harvested August 6 (R.B.),
10 (W.S.), 14 (M.G.)).

Soil	M.G.		R.B.		W.S.	
Fertiliser ...	S	Cy.	S	Cy.	S	Cy.
Dry grain						
No N	15.9	—	17.1	—	4.0	—
0.05 N	14.6	17.0	19.4	18.8	5.8	6.1
0.10 N	19.9	19.5	22.5	22.1	7.8	7.2
0.20 N	19.8	21.6	25.5	26.1	12.5	12.1
0.30 N	20.0	20.2	29.3	28.1	17.1	17.5
0.40 N	19.8	26.0	29.9	30.0	21.6	21.0
0.80 N	18.1	26.0	29.0	32.6	26.9	26.9
Dry grain + straw						
No N	33.2	—	38.6	—	9.0	—
0.05 N	30.7	36.7	43.8	43.3	13.6	14.2
0.10 N	38.6	42.9	51.8	50.1	19.7	18.4
0.20 N	43.8	47.3	57.8	58.6	29.0	27.7
0.30 N	44.8	41.3	66.3	63.2	41.9	40.8
0.40 N	44.9	52.7	67.3	65.9	52.7	48.9
0.80 N	44.5	55.6	68.6	72.3	62.2	56.7
Nitrogen in grain						
No N	0.172	—	0.228	—	0.061	—
0.05 N	0.186	0.237	0.276	0.245	0.080	0.085
0.10 N	0.256	0.243	0.276	0.274	0.087	0.084
0.20 N	0.283	0.317	0.316	0.322	0.142	0.137
0.30 N	0.321	0.298	0.386	0.377	0.186	0.187
0.40 N	0.369	0.423	0.421	0.414	0.237	0.226
0.80 N	0.403	0.612	0.614	0.598	0.390	0.358
Nitrogen in grain + straw						
No N	0.232	—	0.292	—	0.080	—
0.05 N	0.233	0.302	0.351	0.330	0.106	0.112
0.10 N	0.342	0.320	0.354	0.354	0.123	0.131
0.20 N	0.371	0.481	0.421	0.420	0.188	0.184
0.30 N	0.428	0.424	0.513	0.506	0.260	0.254
0.40 N	0.502	0.548	0.567	0.539	0.339	0.318
0.80 N	0.589	0.813	0.863	0.798	0.546	0.470

similar in mineral composition and texture. They were prepared for pot cultures by removing the flints and adding 10 per cent. of washed sand.

The Woburn soil (W.S.) was a sandy loam from Stackyard Field, at the Woburn Experimental Farm, formed on drift over the Lower Greensand. Although the soil at Woburn is generally acid, this particular soil was alkaline. It usually responded markedly to nitrogen, but after storage during the summer of 1928 it became relatively rich in available nitrogen.

The method of these pot experiments was similar to that described by Crowther and Brenchley(5). They were conducted in rather deep earthenware pots (in which nitrification has been shown to be somewhat restricted(1)), and adequate basal dressings of phosphate and potash were always supplied. Except for one treatment in 1929, all fertilisers were uniformly distributed throughout the whole of the soil. Treatments were replicated four- or sixfold. The rates of application of fertiliser, which varied from year to year, are shown in Table V, which gives the yields and total nitrogen contents of the grain and grain + straw obtained with the different treatments.

Throughout these experiments the plants grew well; the early sown barley matured properly and in Rothamsted and Woburn soils gave large responses to added nitrogen. Except in the Millstone Grit soil, the nitrogen percentage on the dry barley grain fell to a minimum with increasing amounts of nitrogen and then began to rise. In each soil the total nitrogen content of the crop, whether barley or mustard, increased linearly with the amount of added nitrogen, giving percentage recoveries which varied between 44 and 67 per cent. in different experiments.

INFLUENCE OF CALCIUM CYANAMIDE ON YIELD AND NITROGEN RECOVERY.

To compare the action of calcium cyanamide and ammonium sulphate over such a range of experiments, instead of examining each comparison separately in terms of the standard error of the yields, it is sufficient to test whether the difference between the two fertilisers increases significantly with the amount of nitrogen given. This makes efficient use of all the pots in the experiment, except those without added nitrogen, and, further, makes it possible to compare not only the results in different years but also those on different crops.

The criterion adopted is to test whether the differences (calcium cyanamide minus ammonium sulphate) in yield or nitrogen content of the crop are proportional to the amounts of nitrogen added. Separate

regression lines, passing through the origin, were fitted for each of the soils of any one experiment with identical and simultaneous treatment of several soils. All the deviations from the regression lines of that experiment were taken together to estimate the standard error of the slopes of the lines.

Table VI. *Summary of differences in yields and in percentage nitrogen recoveries between plants receiving calcium cyanamide (Cy.) and ammonium sulphate (S).*

Significant differences ($P < 0.05$) marked *.

	M.G.	L.C.	R.H.	R.B.	W.S.	Mean standard error
Difference (Cy.-S) in yield of grain + straw, gm. dry matter per gm. nitrogen added						
Barley, 1928	+11.9*	+5.4	-4.3	—	-9.3*	3.76
Mustard, 1928	+0.9	-0.6	-4.2	—	-1.8	2.63
Barley, 1929	+3.1	—	-1.7	-0.3	—	3.41
Barley, 1931	+13.0* (± 4.6)	—	—	+1.5 (± 2.4)	-6.8* (± 0.8)	3.06
Difference (Cy.-S) in percentage recovery of added nitrogen						
Barley, 1928	+18.9*	+8.6	-9.1*	—	-2.2	4.34
Mustard, 1928	+13.1*	+4.8	-5.1	—	-3.9	5.84
Barley, 1929	+13.5*	—	-3.4	-3.9	—	3.66
Barley, 1931	+23.3* (± 6.2)	—	—	-7.1* (± 1.3)	-7.5* (± 1.5)	3.78

All the comparisons of calcium cyanamide and ammonium sulphate in the 1928-31 series of experiments are brought together on this basis in Table VI. The results show a satisfactory consistency. In yield of total dry matter calcium cyanamide twice gave significantly higher yields than ammonium sulphate in the Millstone Grit soil, and the two insignificant results were also positive. In the eight trials on Woburn and Rothamsted soils, ammonium sulphate was significantly above calcium cyanamide twice, and above, but not significantly, five times.

In the percentage nitrogen recovery, a measure of the extent to which the nitrogen applied was utilised by the plants, there were still more consistent differences. In every trial in the Millstone Grit soil calcium cyanamide gave a significantly higher recovery than ammonium sulphate; in the Leagrave soil cyanamide was slightly but insignificantly above ammonium sulphate in each trial; whilst in the Rothamsted and Woburn soils calcium cyanamide was below ammonium sulphate in each of the eight experiments, the difference just reaching the limit for significance in three of the trials. (On account of the larger number of comparisons in 1931, and irregularities in the Millstone Grit soil through fungus attack on the plants, separate standard errors are given for each

of the three soils in that year, as well as the mean for the whole experiment.)

In 1929 single dressings were also applied to the top quarter of the soil in the pots. This difference in the position of the fertilisers had no effect with sulphate of ammonia, but with calcium cyanamide it slightly increased the yield and nitrogen uptake on the Millstone Grit soil and decreased them on the Rothamsted soil. In other words, concentrating the fertiliser in the surface soil tended to magnify the usual differences.

The results of the pot experiments as a whole fall into two groups: (a) calcium cyanamide was more effective than ammonium sulphate in the Millstone Grit soil, and probably also in the Leagrave calcareous soil; (b) it was slightly but consistently poorer in the Rothamsted and Woburn soils.

This grouping of the four soils bears no obvious relation to soil texture or soil reaction.

SOIL NITRATES AND THE DEVELOPMENT OF BARLEY.

An attempt to find a cause for the differences in action of the two fertilisers in the various soils requires some knowledge of the behaviour of the added nitrogen in the soil and of its effect on the growth of the plant. As was explained in the first paper (1), the 1928 barley experiment included a number of pots parallel to the main series, which were arranged with porous tubes down the middle of each pot to allow some of the soil water to be removed for analysis at suitable intervals during the growth of the plants. The treatments studied included the soils without nitrogen and those with the heaviest dressings of calcium cyanamide and ammonium sulphate. The mustard experiment of 1928 had a similar series of pots with porous tubes. The course of development of the barley plants in 1928 and again in 1929 was followed by weekly counts of total shoot numbers, both in the main experiment and in the "porous tube" series. The leaf areas and heights of the mustard plants of 1928 were measured regularly.

A comparison of the rates of formation and death of tillers in the barley experiment of 1928 with the amounts of nitrate in the soil suggested an explanation of the essential differences in the behaviour of calcium cyanamide in the two pairs of soils.

The young barley plant absorbs nitrogen as rapidly as weather and soil conditions allow, and it forms an ever-increasing number of tillers as long as sufficient further nitrogen is forthcoming. Great meristematic activity in the young shoots requires a high nitrogen supply and serves

to form a temporary reserve of nitrogen within the plant. When the nitrogen supply from the soil is exhausted, or at any rate falls below that required to maintain the growth of the large number of young tillers, the smaller ones die as the older and larger ones drain away the accumulated nitrogen. The tiller numbers pass through a maximum and then rapidly fall off. In general, a high initial tillering rate is followed by a high death rate among tillers until the numbers slowly settle down close to the final ear number.

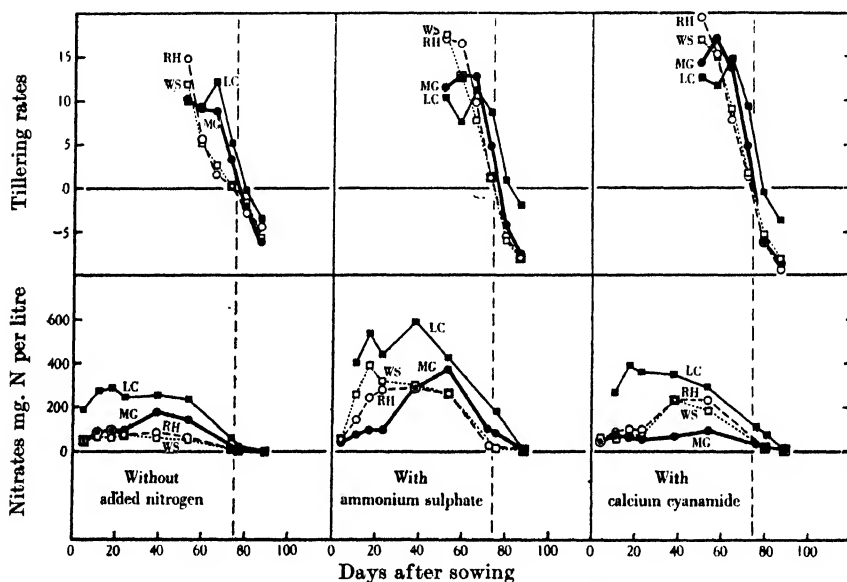


Fig. 1. Mean fortnightly tillering rates (above) and nitrates in soil extracts (below) during the growth of barley, 1928 experiment. Soils: closed circles (M.G.), Millstone Grit; closed squares (L.C.), Leagrave; open circles (R.H.), Rothamsted Hoos Field; open squares (W.S.), Woburn. (H. L. Richardson's data.)

The fortnightly tillering rates were chosen to illustrate the rate of development in the 1928 and 1929 barley experiments, and, in order to use all of the available replicates, the means for each soil in the series with added nitrogen were weighted according to the amount of nitrogen given. The fortnightly mean tillering rates per pot are plotted at weekly intervals in Fig. 1 for the four soils grouped according to treatment. On the same time scale the nitrate concentrations in similar soils with the heaviest nitrogen dressings are also given. (The individual pot results for these analyses were shown in Fig. 5 of the first paper (1).)

The tillering rate curves bring out clearly the essential differences between the four soils. In the *unmanured series* the Rothamsted and

Woburn soils gave high initial tillering rates, which fell off very rapidly—the second value plotted being but half of that one week earlier. Maximum tiller numbers (zero tillering rate) were reached 75 days after sowing, just at the time when the soil nitrates fell below the limits for analysis (less than 10 parts N per million in the soil extract). The other two soils gave somewhat lower initial tillering rates but maintained similar rates for the next two weeks, and were still tillering actively when the plants in Rothamsted and Woburn soils had ceased tillering. The Millstone Grit and Leagrave soils had much higher nitrate contents at the time of maximum tillering than the Rothamsted and Woburn soils, and still had small supplies of nitrate when those in the Rothamsted and Woburn soils were exhausted.

These relationships are shown still more clearly in the pots receiving *ammonium sulphate*. The plants in Rothamsted and Woburn soils had higher initial tillering rates and much higher rates one week later than the corresponding ones with no nitrogen, but they reached zero tillering rates at the same time as the unmanured series (75 days after sowing). The nitrates too were exhausted at this date. The ammonium sulphate did not affect early tillering rates for the Leagrave soil and only slightly increased those for the Millstone Grit soil. At 75 days after sowing, when the plants in the Rothamsted and Woburn soils had ceased tillering, those in the two richer soils were still tillering actively. The plants in the Leagrave soil at 75 days were tillering almost as rapidly as during the three preceding weeks and the nitrate content of the soil was still high—higher in fact than at any stage in the unmanured Rothamsted and Woburn soils. The Millstone Grit soil gave about half the tillering rate and also half of the nitrate content of the Leagrave soil. In both the Leagrave and the Millstone Grit soils tillers were lost more slowly (lower negative tillering rates) than in the other two soils.

These parallelisms between tillering rates near the time of maximum tiller numbers and the disappearance of nitrates appear to justify the view that, under the conditions of these experiments, changes in tiller rates may be taken to reflect changes in available nitrogen supply. Doubtless the absolute magnitude of the tillering rate depends on many other factors, both of weather and soil, but in comparisons between fertilisers supplying equal amounts of nitrogen in parallel experiments on a single soil there should be no serious error in ascribing observed differences in rate of tillering to differences in the amounts of nitrogen available to the plant.

When the action of *calcium cyanamide* is examined with this in mind,

the outstanding difference between the four soils tested lies in the inhibition of nitrification—strictly, nitrate-accumulation—which was slight and of relatively short duration in the Rothamsted and Woburn soils and more marked and prolonged in the other two. The effect was so great with the Millstone Grit soil that throughout the 1927 barley experiment the nitrate concentration with calcium cyanamide was not only much below that with an equivalent amount of sulphate of ammonia, but considerably below that of the unmanured soil. Reference to Fig. 1 shows that in spite of the great reduction of nitrate concentration in the Millstone Grit soil through calcium cyanamide, the early rates of tillering were actually higher with calcium cyanamide than with ammonium sulphate. This shows that in pots treated with calcium cyanamide the nitrate content does not measure the available nitrogen.

In Table VII comparisons of calcium cyanamide and ammonium sulphate are made in terms of the mean increases in shoot numbers per pot over a three-weeks' interval in the barley experiments of 1928 and 1929. In four of the eight experiments calcium cyanamide gave significantly higher tillering rates than ammonium sulphate, and in the other four the differences, though insignificant, were in the same direction.

Table VII. *Mean increase in tiller numbers per pot from the 45th to the 66th day after sowing.*

Soil	1928		1929	
	Ammonium sulphate	Cyanamide	Ammonium sulphate	Cyanamide
Leagrave	16.0	20.5*	—	—
Millstone Grit	20.5	24.6*	21.6	26.6*
Rothamsted Hoos	25.3	25.5	27.3	28.2
Rothamsted Broadbalk	—	—	23.7	26.6*
Woburn	24.4	25.2	—	—
Standard error	0.98		0.87	

Mean of both years and all soils: S=22.7; Cy.=25.6*. S.E.=0.39. Asterisks denote significant increases with calcium cyanamide.

The differences between calcium cyanamide and ammonium sulphate in their effects on soil nitrate and rates of tillering are given directly in Fig. 2 for the barley experiments of 1928 and 1929. In the Rothamsted and Woburn soils the excess nitrate accumulation from ammonium sulphate became small before active tillering started, and the slight initial superiority from calcium cyanamide in tillering rates was soon lost.

In the Leagrave and Millstone Grit soils the ammonium sulphate gave considerably more nitrate than calcium cyanamide throughout the period of active tillering. In both of these soils calcium cyanamide gave

a marked superiority in initial tillering rate, lasting three or four weeks. The differences after maximum tiller numbers were somewhat erratic, though of the same general form in the two years for the two soils examined twice. A high initial rate is usually followed by a high death rate among tillers, but the actual course of the curve must depend on the relative sizes of the tillers and many other factors.

The tillering rates up to the period of maximum tiller numbers show differential effects of the same type as those in the final yields of barley.

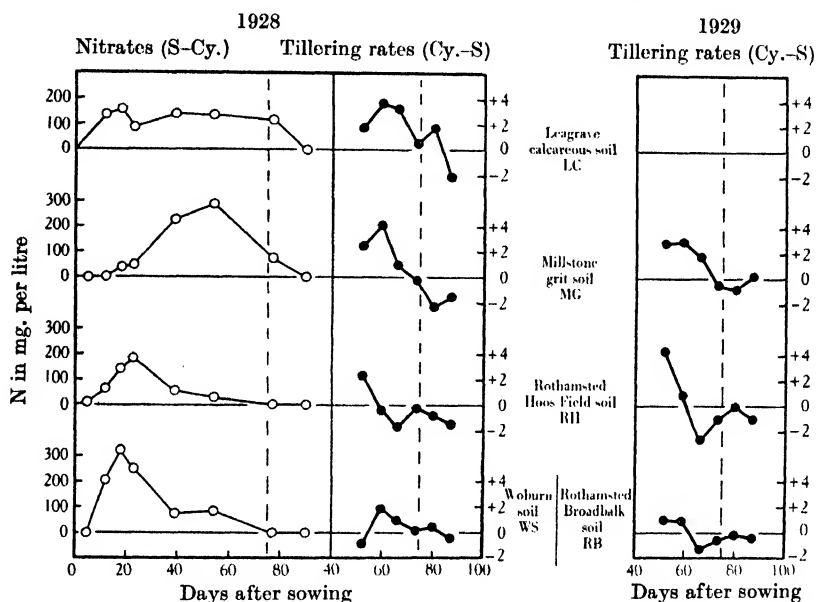


Fig. 2. Excess of nitrate in soil extract from ammonium sulphate (S) over calcium cyanamide (Cy.), in the 1928 barley experiment, and excess of fortnightly tillering rates per pot from calcium cyanamide over ammonium sulphate in the 1928 and 1929 barley experiments. (H. L. Richardson's data.)

It seems safe to attribute these differences in a large measure to the higher availability of the soil nitrogen during the period of active tillering, and to conclude that in the early stage of growth calcium cyanamide yielded more available nitrogen than ammonium sulphate in the Millstone Grit soil and probably in the Leagrave soil, but less in the Rothamsted and Woburn soils.

It was shown in the first paper, and it will be demonstrated again from other experiments that follow, that the lower nitrate content of soils with calcium cyanamide is balanced by an almost equivalent amount of ammonia. The nitrate differences (S-Cy.) in Fig. 2 may thus be re-

garded as measuring the extra concentration of ammonia nitrogen in the soil treated with calcium cyanamide. We advance the hypothesis that the extra tillering from calcium cyanamide in the soils in which initial nitrification is more markedly retarded, is to be ascribed to the greater availability to the plant of ammonia nitrogen as compared with nitrate nitrogen.

At the time these experiments were made this hypothesis was put forward with some diffidence, for it seemed directly counter to commonly accepted views on the relative availability of nitrogen fertilisers. There is, however, much plant physiological evidence to support it, and recent work from a number of sources, reviewed elsewhere by one of us (6), has shown that under suitable conditions ammonia nitrogen is no less available to plants than nitrate nitrogen. Indeed, in water culture experiments and in sand cultures with flowing nutrient solutions, at a pH approaching neutrality, young plants are found to absorb ammonia preferentially to nitrate, provided that the calcium and carbohydrate supplies are adequate.

In the soil the absorption of ammonium ions by colloids introduces a disturbing factor, but the results of our experiments show that the absorbed ammonia is still so readily available to plants that it may cause more rapid tillering than nitrate nitrogen. Field experiments would not necessarily show this effect as clearly as pot experiments, because of the difficulty of securing a thorough and uniform distribution of added ammonia nitrogen.

SOIL NITRATE AND AMMONIA AND THE DEVELOPMENT OF MUSTARD.

The mustard plants in the autumn experiments of 1928 developed much more rapidly than the barley in the corresponding spring experiments. The porous tube extracts showed that vigorous nitrogen uptake occurred before nitrification was complete in some of the soils. The nitrification of calcium cyanamide was again slower than that of ammonium sulphate, and unmanured pots lost their nitrate ten or more days before those with added nitrogen. The rates of growth in height roughly followed the initial nitrate contents of the soils, except that, as with barley, inhibition of nitrification by calcium cyanamide did not cause the plants to fall behind those with ammonium sulphate.

Deductions about nitrogen uptake from tillering curves and about ammonia contents from porous tube samplings of the soil water are necessarily indirect; they were therefore supplemented by experiments

in which pots receiving each treatment were removed for analysis of both soil and plant at suitable stages. It is difficult to obtain adequate replication in such experiments, for apart from restrictions on the greenhouse accommodation available, the soil analyses must be made very soon after the thorough aeration to which the soil is exposed during sampling.

In an additional mustard experiment of 1928, parallel with the main series, the porous tube method of following nitrate levels was combined with periodic removal of pots for sampling the whole of the soil and the plants. The Millstone Grit soil was used, with no nitrogen and with 0.424 gm. nitrogen per 10 kg. pot as ammonium sulphate or as calcium cyanamide. The experiment began with six fold replication, which was gradually reduced as pots were removed for sampling.

Nitrate determinations were made on the porous tube extracts from individual pots at intervals of three or four days. During the early stages there was sufficient replication for the variation between pots to be measured. The percentage standard errors for a single determination, calculated on a logarithmic basis, were:

Determination no.	1-3	4-6	7-8	9-12	13-14
Days after sowing	2, 8, 12	16, 20, 23	27, 30	35, 40, 44, 48	52, 57
No. of pots per treatment	6	5	4	3	2
Standard error %	3.1	4.0	2.8	5.0	—

The uniformity of the pots and the analyses is thus satisfactory. The results of these analyses are plotted in Fig. 3 using for each treatment double lines obtained by joining points representing the mean ± 1.5 times the standard error of the mean. Except where the boundary lines for two treatments overlap, the differences in mean nitrate content for the two treatments may be taken as significant. It will be seen that over the great part of the range the curves are widely separated. Calcium cyanamide retarded nitrification for 30 days, and the nitrate concentration then rose suddenly, so that for the period from 35 to 48 days after sowing it was above that from ammonium sulphate. The general rise in nitrate concentration at 52 days coincided with the removal of the pots from an open cage into the greenhouse, where the average temperature was warmer. The results agreed well with those obtained in Millstone Grit soil in the main experiment with four soils.

Fig. 3 also gives the results of soil and plant analyses from single pots per treatment taken down at successive stages. Since single pots were concerned, isolated differences have little value, but the trend of several determinations may give useful information. The results show clearly

that, in the soil, the ammonium content from calcium cyanamide remained high as long as nitrification was inhibited, but the sum of the ammonia and nitrate nitrogen in the calcium cyanamide pots was slightly below that from sulphate of ammonia. In the first four weeks, while the plants were small, those with calcium cyanamide had a slightly but consistently higher percentage of nitrogen in the dry matter than those from ammonium sulphate (Means: calcium cyanamide 6.24 per cent.; ammonium sulphate 6.05 per cent.; no added nitrogen 5.59 per cent.). The extra nitrogen appears to have encouraged growth, for, subsequently,

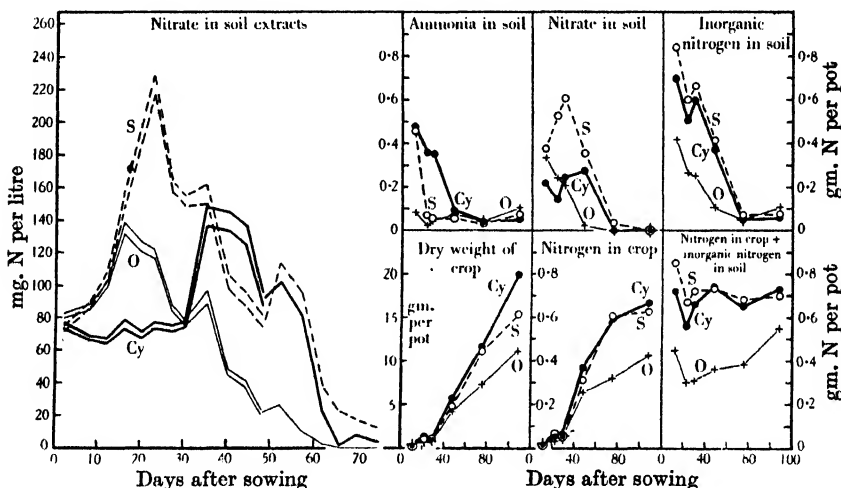


Fig. 3. Inorganic soil nitrogen and nitrogen in crop during the growth of mustard, in the 1928 experiment, with ammonium sulphate (S), calcium cyanamide (Cy.), and no added nitrogen (O). Left: nitrate in porous tube soil extracts, as mg. N per litre. Right: nitrogen in gm. per pot. as ammonia and nitrate in soil and as total nitrogen in crop. (H. L. Richardson's data.)

the plants with cyanamide had more dry matter but no higher nitrogen percentage than the ammonium sulphate plants. The leaf area was also somewhat larger with calcium cyanamide.

These results again suggest that the low initial soil nitrate content with calcium cyanamide, as compared with ammonium sulphate, is no handicap to the plant in obtaining its nitrogen, but that the low nitrate level is counterbalanced by the great availability of the ammonia remaining.

The nitrogen balance throughout this experiment illustrates, and partially explains, the low recovery of added nitrogen which is generally found in pot and field experiments. In the first three samplings almost

the whole of the added ammonium sulphate (0.42 gm. nitrogen per pot) could be accounted for in the nitrogen present in the plant and in inorganic forms in the soil. Subsequently the sum of plant and inorganic soil nitrogen in the pots without added nitrogen increased steadily, whilst that for pots with nitrogenous fertilisers remained practically constant. The recovery of added nitrogen steadily decreased; in the main mustard experiment of 1928 about one-half of the added nitrogen was recovered in the final crop, although only traces remained as nitrate in the soil. Similar results have been obtained in many other pot culture experiments. When no nitrogenous fertiliser is given, there is a slow production and uptake of available nitrogen from the soil during the later stages of growth, but when a nitrogenous fertiliser is added, this extra nitrogen is not liberated.

SUMMARY.

1. In pot culture experiments with barley and mustard conducted in several soils over a number of years, the yield differences between calcium cyanamide and ammonium sulphate were generally small. Calcium cyanamide gave slightly poorer results than ammonium sulphate in soils with high responses to added nitrogen but definitely better ones in soils which contained much available nitrogen and in which calcium cyanamide greatly retarded nitrification.

2. The pot culture experiments confirmed the conclusion from earlier laboratory work that in normal soils calcium cyanamide was converted through urea into ammonia within a few days. Nitrate accumulation was less complete and slower from calcium cyanamide than from ammonium sulphate. In one soil the nitrogen from calcium cyanamide remained as ammonia for several weeks, the nitrate content being below that even of unmanured soil.

3. Where the ammonia from calcium cyanamide remained for several weeks, tillering of barley was more rapid and the final yields and nitrogen contents were higher than with ammonium sulphate. It is suggested that the young barley plant utilises ammonia nitrogen more readily than nitrate, provided the ammonia is thoroughly distributed through the soil. It is clear that the amount of nitrate obtained in nitrification tests should not be used as a measure of the relative values of calcium cyanamide and other nitrogenous fertilisers.

REFERENCES.

- (1) CROWTHER, E. M. and RICHARDSON, H. L. *J. agric. Sci.* (1932), **22**, 300.
- (2) MUKERJI, B. K. *J. agric. Sci.* (1932), **22**, 335.
- (3) CROWTHER, E. M. and RICHARDSON, H. L. *Emp. J. exp. Agric.* (1935) (in the press).
- (4) DAWSON, R. B. Thesis for the Degree of M.Sc. (University of London), 1927.
- (5) CROWTHER, E. M. and BRENCHLEY, W. E. *J. agric. Sci.* (1934), **24**, 156.
- (6) CROWTHER, E. M. *Rep. Progr. Appl. Chem.* (1930), **15**, 475; (1933), **18**, 530.

(Received August 3rd, 1934.

THE FORMATION OF LATIN SQUARES FOR USE IN FIELD EXPERIMENTS

F. YATES

(Rothamsted Experimental Station)

1. *Introduction*

REPLICATION was originally introduced into agricultural field trials with the object of securing greater accuracy by distributing the different treatments more evenly over the field and so balancing out the fertility differences. The estimation of the error to which the results were subject did not at first receive much consideration, but with the evolution of a logical basis for inductive reasoning it became apparent that wholly systematic arrangements of plots in field trials were incapable of furnishing valid estimates of error, without which it was impossible to draw any completely objective conclusions from the results. In order to obtain a valid estimate of error it is essential that there shall be some element of randomness in the arrangement of the plots, and that any restrictions which are imposed for the purpose of eliminating soil heterogeneity shall be such that their effects on the reduction of error can be clearly isolated by the procedure of the analysis of variance. The simplest type of arrangement fulfilling these conditions is that of randomized blocks, where the plots are arranged so that every treatment occurs an equal number of times in every block, the random element in the arrangement being very simply introduced by assignment of the treatments to the different plots within each block entirely at random.

For many experiments randomized block arrangements are eminently suitable, being very flexible and capable of a wide variety of applications. On the other hand the Latin square, where each treatment occurs once and once only in each row and each column, though by no means so flexible, may be expected on the average to eliminate soil heterogeneity more completely, at any rate when the size of the square is not too large, since soil variations in two directions at right angles affect all treatments equally. Such elimination is peculiarly attractive in agriculture because most agricultural operations are carried out in long strips, and by arranging that these strips shall be parallel to one of the sides of the square variation from this source is eliminated.

The value, as a means of eliminating fertility differences, of square arrangements of plots, satisfying the conditions of the Latin square, was early recognized. When first introduced, however, the importance of an unbiased estimate of error was not realized, and the arrangements adopted were all systematic, usually of some specially simple type, or alternatively of a type which was believed to be capable of removing most completely the soil differences ordinarily existing. The term Latin square, used by Euler [1] in his study of the enumeration of the different possible square arrangements, was introduced into agricultural science to designate a square chosen at random out of the totality of these arrangements.

Striking practical confirmation of the failure of systematic square arrangements to give a valid estimate of error was afforded by the work of Tedin [2]. He took twelve 5×5 Latin-square arrangements, four of them systematic, and applied them to data from eight uniformity trials collected from various sources, which provided in all 91 separate squares of 25 plots. The treatment and error sums of squares were computed for each of the arrangements. Since the treatments were dummy the error sum of squares (12 degrees of freedom) should on the average be three-quarters of the total sum of squares after eliminating rows and columns (16 degrees of freedom), and significant divergence from this fraction would be an indication of a biased estimate of error. Two of the arrangements chosen were knight's move patterns, and these gave an average fraction of 0.7720, significantly higher than 0.75, indicating that such arrangements are in general more accurate than the average of all random arrangements, but appear to be less accurate. This accords with the claims advanced for this type of design. The actual amount of the bias introduced may best be indicated by the number of times the result may be expected to be judged significant on a 5 per cent. basis, this, Tedin concludes, being 4.2 times out of 100 in the squares under consideration. The data are not extensive enough, however, for this value to be considered as at all accurately determined.

The other pair of systematic arrangements were of the diagonal pattern (the second square of Fig. 2 and the same square rotated through a right angle). These arrangements are chosen as an example for discussion in the next section. The particular type of fertility correlation there considered does not appear to give rise to any particular bias in the error, though there may be an excess of very high and low values. Tedin, however, reasoning on the proximity or dispersion of similarly treated plots, concludes that this pair of arrangements should have a low average estimate of error. The actual average value of the fraction of the sum of squares allotted to error, 0.7291, supports his conclusion, though the difference from 0.75 is not fully significant. There is also some slight evidence of an excess of very low values in one of the arrangements.

In contradistinction to the two systematic arrangements tested, the distribution of z in the eight Latin squares (728 values) compared excellently with expectation.

2. *Conditions for an Unbiased Estimate of Error*

The element of randomization may be introduced into Latin-square arrangements by making a selection of one square from a whole set of squares. It remains to consider what particular types of set will give an estimate of error which is unbiased when averaged over all the squares of the set.

The z distribution, on which the tests of significance depend, is established in the first place on the assumption that the yields are an uncorrelated sample from a normally distributed population. (The word sample here implies the whole set of yields that go to form a single experiment.) It requires an infinity of experiments to generate the distribution of the statistic z .

The procedure of the analysis of variance enables correlations between whole rows and columns to be eliminated. The z distribution will still hold, whatever the Latin-square arrangement adopted, if the residuals (i.e. the remainders after deducting from each plot the general mean and the deviations from the general mean of the means of the row and column in which it occurs) form a sample from a normal population without any correlations except those introduced by the conditions that the sum of the residuals of each row and each column is necessarily zero.

Actually the assumption of no correlation between the residuals of neighbouring plots is unjustified. A complex and unknown system of correlations must be assumed, these correlations being what is left of the inherent correlations between neighbouring plots after the removal of row and column effects. As an example we may consider a system of experiments in which the fertility of the land is distributed in strips running diagonally across the lay-out of the square. In this case there will be a positive correlation between the residuals of the plots lying on the system of parallels crossing the square in this direction, i.e. between the plots bearing the same number in Fig. 1. In the limiting case, when

1	2	3	4	5	6
2	3	4	5	6	7
3	4	5	6	7	8
4	5	6	7	8	9
5	6	7	8	9	10
6	7	8	9	10	11

FIG. 1.

A	B	C	D	E	F
B	C	D	E	F	A
C	D	E	F	A	B
D	E	F	A	B	C
E	F	A	B	C	D
F	A	B	C	D	E

FIG. 2.

the correlations are perfect, the residuals of all plots bearing the same number will be equal in every sample, and since the sums of the residuals of every row and column are zero the residuals of plots 1 will equal the residuals of plots 7, &c.

If in this example a Latin square of the pattern shown in Fig. 2 be chosen as the experimental lay-out in the series of experiments, it is clear that the treatment sum of squares will tend to be too large and the error sum of squares too small, and in the limiting case where the correlation is perfect, the error sum of squares will always be zero and consequently z will always be infinite. Conversely, if the arrangement chosen be that of Fig. 2, but turned through a right angle, the treatment sum of squares will tend to be too small, and in the limiting case it will always be zero, and z will be negatively infinite.

This example provides a simple illustration of how, given a certain system of correlations between the residuals, the use of a single Latin square will give estimates of error which are biased. If, however, a suitably chosen set of Latin squares is used, such bias in the estimate of error can be entirely eliminated.

The simplest set of squares which will eliminate the bias in the estimate of error, whatever the system of correlation between the residuals, is that obtained by permuting all the rows except the first of any chosen square in all possible ways. In such a set of squares every pair of plots not in the same row or column receives like treatments equally frequently.

In the set of squares generated from that shown in Fig. 2, for example, A of the second column will occupy each of the last five places in the column 24 times, so that the first plot of the first column has like treatment to each of the other plots of the second column 24 times. The second plot of the first column will receive treatment B 24 times and will then have like treatment with the first plot of the second column. It will receive treatment C 24 times, and of these 24 times C will fall in the last four places of the second column 6 times each. The same occurs with D, E, and F, and therefore this plot is associated with each of the last four plots of the second column 24 times.

The absence of bias in the error can be shown as follows. Let the residuals of a single $n \times n$ experiment, after correcting for rows and columns, be given by Table I.

TABLE I

x_{11}	x_{12}	x_{13}	.	.	x_{1n}
x_{21}	x_{22}	x_{23}	.	.	x_{2n}
x_{31}	x_{32}	x_{33}	.	.	x_{3n}
.
x_{n1}	x_{n2}	x_{n3}	.	.	x_{nn}

The sums of the residuals of every row and every column are necessarily zero. If the totals of the residuals for the n treatments are $T_A, T_B, T_C, \dots T_K$, then the sum of squares due to treatments is

$$\frac{1}{n} (T_A^2 + T_B^2 + T_C^2 + \dots + T_K^2).$$

On permuting the last $n-1$ rows in all the $(n-1)!$ possible ways, $(n-1)!$ such treatment sums of squares will be obtained. Since every pair of plots not in the same row and same column have like treatment the same number of times, and the square of each treatment total when multiplied out contains n squares of residuals and $\frac{1}{2}n(n-1)$ pairs of products, it follows that the total of all the $(n-1)!$ treatment sums of squares must reduce to

$$\frac{(n-1)!}{n} \cdot Sx_{rs}^2 + \frac{(n-1)!}{n(n-1)} Sx_{rs}x_{tu}, \quad t \neq r, u \neq s,$$

where the first summation represents the sum of all squares of residuals and the second summation twice the sum of the products of residuals of all pairs of plots not in the same row or column.

The sum of the products of any residual, say x_{11} , with all the residuals not in the same row or column, is equal to the square of that residual, for $x_{22} + x_{23} + \dots + x_{2n} = -x_{21}$, &c., so that the required sum is equal to $-x_{11}(x_{21} + x_{31} + \dots + x_{n1})$, i.e. x_{11}^2 . Hence the last summation in the above expression is equal to Sx_{rs}^2 , and therefore the mean treatment sum of squares reduces to

$$\frac{1}{n-1} Sx_{rs}^2.$$

This corresponds to $n-1$ degrees of freedom, whereas the total sum of squares of the residuals after correcting for rows and columns, Sx_{rs}^2 , corresponds to $(n-1)^2$ degrees of freedom. There is consequently no bias in the treatment sum of squares if the whole set of Latin squares is applied to the results of a single experiment. There can therefore equally be no bias if the whole set of squares is applied to each of the whole population of experiments, or, since this population is infinite, if a single square selected at random from the set is applied to each member of the whole population.

The permutation of the last $n-1$ rows removes all bias from the estimate of error, but since the first row is the same for all squares, individual treatments are still not free from bias if a single experiment is considered, or from correlation if we consider the whole population of experiments. Such bias and correlation can be eliminated by permuting all the letters of the square. Alternatively, the elimination can be performed just as effectively by permuting all the columns.

Although the employment of such a method of randomization will eliminate the bias due to error and any correlation between treatments, it appears that with a given system of correlations between the residuals, the z distribution will not be exactly realized. Since the greatest bias in z is introduced when the pattern of the square coincides most nearly with the correlation pattern, or cuts across it most completely, as in the example discussed above, it would seem theoretically preferable to choose a square at random from all the possible squares of given size, since with such a choice the maximum bias will occur less frequently than if the square is always chosen from a set as defined above, which happens to contain a square coinciding most nearly with the correlation pattern. The point is largely theoretical, for in agricultural experiments the correlation system of an infinity of experiments can hardly be very large, or of the type that will coincide at all completely with any possible Latin square.

Instead of considering a whole population of experiments, we may consider the z distribution which would be obtained if a set of arrangements be applied to a single experiment. Such a distribution is necessarily discontinuous, for the number of different values of z is some fraction of the number of arrangements in the set. In order that the distribution shall approximate to a continuous distribution the number of arrangements included in the set must be large. This is the basis of the test made by Eden and Yates [3] on 256 height measurements of wheat, which displayed marked departure from the normal form of distribution. The data were arranged in eight blocks of four values (each value being the sum of eight measurements) and a sample of 1,000 arrangements taken at random from the whole $(4!)^8$ possible arrangements, the z being computed for each arrangement. There are $(4!)^7$ discrete values of z , and it is shown that the sample of 1,000 from this discontinuous population conforms satisfactorily with the theoretical z distribution for an infinite population of normally distributed data.

In order that such a distribution shall approximate to a continuous distribution the number of arrangements included in the set must be

large, and this may be regarded as a further point in favour of using a set of squares as large as conveniently possible. In the case of 5×5 squares, for example, the permutation of all the rows, except the first, of a given square will only give twenty-four different values of z , whereas the utilization of all squares will give 1,344 such values.

All possible squares up to 6×6 have been enumerated and are easily presentable [4]; they are therefore illustrated in the next section. A single square of each size from 7×7 to 12×12 , is also given. From these typical squares experimental arrangements may be derived as required.

3. Typical Squares

The most extensive set of squares that can be easily derived from a single square is that generated by the permutation of all rows, all columns and all letters. This type of permutation, which we have styled a *transformation* in the enumeration of the 6×6 squares [4], is the basis of the presentation of the 5×5 and 6×6 squares. The greatest number of $n \times n$ squares that can possibly belong to any transformation set is clearly $(n!)^3$, since the rows, columns, and letters may all be permuted independently in $n!$ ways (including no change), but all transformations of a given square do not necessarily give different squares, so that the actual number in the set may be very much less than this. In the case of 6×6 squares, for example, the greatest number of squares in any one set is $\frac{1}{4}(6!)^3$ and the least number $_{216}(6!)^3$.

Although all transformations do not in general give different squares, every square of the set (including the original square) must be generated an equal number of times, when all the $(n!)^3$ transformations (including no change) are made. Thus in the case of 6×6 squares every square of a set containing $\frac{1}{4}(6!)^3$ squares must be generated four times. It follows that if a transformation be chosen at random from all the possible $(n!)^3$ transformations, this transformation, when applied to any given square of a set, will generate a square which is a random selection from all possible squares in the set. In order to make a random selection from the $(n!)^3$ transformations it is only necessary to choose some new random order for the rows and columns and a random substitution for the letters (by numbered cards, balls, &c., or a table of random numbers [5]).

The fraction $1/n!(n-1)!$ of all the $n \times n$ Latin squares will be what are called reduced squares, i.e. squares with the first row and first column in the given order A.B.C.D.... Each transformation set contains the same fraction of reduced squares. From each reduced square $n!(n-1)!$ squares can be generated by permuting all the rows except the first, and all the columns, or alternatively, all the rows except the first, and all the letters, and either of these generating processes, when applied to all reduced squares, will generate all possible squares.

The 3×3 squares are all included in a single transformation set, which contains one reduced square, so that there are $3!2!$ or twelve 3×3 squares in all. The reduced square is illustrated in Fig. 3. A random selection

from all the squares may be made by permuting at random the last two rows and all the columns or letters.

A B C
B C A
C A B

FIG. 3. The 3×3 squares.

The 4×4 squares form two transformation sets, one containing three reduced squares, all of which are illustrated in Fig. 4 (a), and the other a single reduced square, Fig. 4 (b). A random selection from all the squares may be made by selecting one of the reduced squares at random and permuting the last three rows and all the columns or letters.

A B C D	A B C D	A B C D	A B C D
B A D C	B C D A	B D A C	B A D C
C D B A	C D A B	C A D B	C D A B
D C A B	D A B C	D C B A	D C B A
(a)	(a)	(a)	(b)

FIG. 4. The 4×4 squares.

The 5×5 squares also form two transformation sets, one containing 50 and the other 6 reduced squares. Space does not permit us to set out all these 56 reduced squares in full, and a single example of each transformation set is therefore given, in Fig. 5, from which all squares of the set may be generated by the permutation of all rows, all columns, and all letters.

To make a random selection from all possible 5×5 squares with an equal probability of obtaining any one square, it is first necessary to select one or other of the transformation sets, in such a manner that the probability of selection is proportional to the number of squares in the set. This may be done most simply by selecting a number at random from the numbers 1-56, and choosing the first set when a number between 1 and 50 is obtained, and the second when a number between 51 and 56 is obtained, as indicated by the key numbers printed below the squares.

A B C D E	A B C D E
B A D E C	B C D E A
C E A B D	C D E A B
D C E A B	D E A B C
E D B C A	E A B C D
1-50	51-6

FIG. 5. The 5×5 squares.

The 6×6 squares, Fig. 6, form 22 transformation sets, comprising in all 9,408 reduced squares. Ten of these sets form 5 pairs of sets, such that all squares of one set of the pair are the conjugates of the squares in the other set. (One square is said to be the conjugate of another if the rows of one square, taken in order, correspond to the columns of the other, also taken in order.) These pairs of sets are illustrated by a single

example each, but are distinguished from those sets which include conjugates by being given two sets of key numbers. Since all the squares of one of a conjugate pair of sets, when rotated through a right angle, give the squares of the other set, the agriculturist can hardly be concerned with the distinction between a square and its conjugate, for the orientation of an experiment is itself usually arbitrary. The purist may satisfy himself by performing the rotation if a number included in the lower group of key numbers is obtained.

Apart from the occurrence of these conjugate pairs of sets, the method of selection of a square at random from all the possible 9,408. 6! 5! 6×6 squares is identical with that already described in the case of the 5×5 squares.

I

A B C D E F
B C F A D E
C F B E A D
D E A B F C
E A D F C B
F D E C B A

0001-1080
1081-2160

II

A B C D E F
B C F E A D
C F B A D E
D E A B F C
E A D F C B
F D E C B A

2161-3240

III

A B C D E F
B A F E C D
C F B A D E
D C E B F A
E D A F B C
F E D C A B

3241-4320

IV

A B C D E F
B A E F C D
C F B A D E
D E A B F C
E D F C B A
F C D E A B

4321-5400

V

A B C D E F
B A E C F D
C F B A D E
D E F B C A
E D A F B C
F C D E A B

5401-5940
5941-6480

VI

A B C D E F
B A F E C D
C F B A D E
D E A B F C
E C D F B A
F D E C A B

6481-7020

VII

A B C D E F
B C D E F A
C E A F B D
D F B A C E
E D F C B A
F A E C D B

7021-7560

VIII

A B C D E F
B A E F C D
C F A E D B
D C B A F E
E D F C B A
F E D B A C

7561-7920
7921-8280

IX

A B C D E F
B A E F C D
C F A B D E
D E B A F C
E D F C B A
F C D E A B

8281-8640

X

A B C D E F
B C F A D E
C F B E A D
D A E B F C
E D A F C B
F E D C B A

8641-8820

XI

A B C D E F
B C A F D E
C A B E F D
D F E B A C
E D F C B A
F E D A C B

8821-8940
8941-9060

XII

A B C D E F
B C A E F D
C A B F D E
D E F B A C
E F D A C B
F D E C B A

9061-9180

XIII						XIV						XV					
A	B	C	D	E	F	A	B	C	D	E	F	A	B	C	D	E	F
B	C	A	F	D	E	B	C	A	E	F	D	B	A	F	E	D	C
C	A	B	E	F	D	C	A	B	F	D	E	C	D	A	B	F	E
D	F	E	B	A	C	D	F	E	B	A	C	D	F	E	A	C	B
E	D	F	A	C	B	E	D	F	C	B	A	E	C	B	F	A	D
F	E	D	C	B	A	F	E	D	A	C	B	F	E	D	C	B	A
9181-9240						9241-9280						9281-9316					
												9317-9352					
XVI						XVII											
A	B	C	D	E	F	A	B	C	D	E	F						
B	A	E	C	F	D	B	C	A	F	D	E						
C	E	A	F	D	B	C	A	B	E	F	D						
D	C	F	A	B	E	D	E	F	A	B	C						
E	F	D	B	A	C	E	F	D	C	A	B						
F	D	B	E	C	A	F	D	E	B	C	A						
9353-9388						9389-9408											

FIG. 6. The 6×6 Latin squares.

No enumeration has as yet been made of squares larger than 6×6 . In Fig. 7 we give six squares, with sides from 7 to 12, from which any square of the transformation sets which contain them may be generated by the permutation of all rows, columns, and letters amongst themselves. These transformation sets, or even the smaller sets generated by the permutation of rows and columns, or either and letters, will give sets of squares amply large enough to serve all agricultural purposes.

A B C D E F G
B D E F A G C
C G F E B A D
D E A B G C F
E C B G F D A
F A G C D E B
G F D A C B E

 7×7

A B C D E F G H
B C A E F D H G
C A D G H E F B
D F G C A H B E
E H B F G C A D
F D H A B G E C
G E F H C B D A
H G E B D A C F

 8×8

A B C D E F G H I
B C E G D I F A H
C D F A H G I E B
D H A B F E C I G
E G B I C H D F A
F I H E B D A G C
G F I C A B H D E
H E G F I A B C D
I A D H G C E B F

 9×9

A B C D E F G H I J
B G A E H C F I J D
C H J G F B E A D I
D A G I J E C B F H
E F H J I G A D B C
F E B C D I J G H A
G I F B A D H J C E
H C I F G J D E A B
I J D A C H B F E G
J D E H B A I C G F

 10×10

A	B	C	D	E	F	G	H	I	J	K
B	A	J	I	D	C	F	K	H	G	E
C	K	H	A	B	I	J	F	D	E	G
D	C	G	J	I	K	E	B	F	A	H
E	J	B	G	K	H	D	C	A	I	F
F	E	I	C	G	A	K	J	B	H	D
G	F	D	B	H	J	A	I	E	K	C
H	I	K	F	A	D	B	E	G	C	J
I	D	E	H	J	B	C	G	K	F	A
J	G	A	K	F	E	H	D	C	B	I
K	H	F	E	C	G	I	A	J	D	B

11 × 11

A	B	C	D	E	F	G	H	I	J	K	L
B	L	G	C	D	J	K	E	H	A	F	I
C	K	A	B	F	L	I	D	G	H	J	E
D	F	I	A	L	E	C	G	J	B	H	K
E	D	F	G	J	K	A	L	C	I	B	H
F	H	K	E	G	C	D	B	A	L	I	J
G	I	D	F	K	H	J	A	L	C	E	B
H	E	L	J	C	A	B	I	K	D	G	F
I	J	B	L	H	G	F	K	D	E	A	C
J	C	E	K	A	I	H	F	B	G	L	D
K	G	J	H	I	B	L	C	E	F	D	A
L	A	H	I	B	D	E	J	F	K	C	G

12 × 12

FIG. 7.

4. Summary

1. The conditions which must be fulfilled in selecting Latin-square arrangements for agricultural field trials, if an unbiased estimate of error is to be obtained, are discussed.

2. Examples of squares up to size 12×12 are given, from which experimental arrangements may be derived by simple processes of permutation. All squares up to size 6×6 have been enumerated elsewhere, and the totalities of these squares are presented here in compact form.

REFERENCES

1. L. EULER, Recherches sur une nouvelle espèce de quarrés magiques. Verh. v. h. Zeeuwsch Genootsch. der Wetensch. Vlissingen, 1782, 9, 85-239.
2. O. TEDIN, The Influence of Systematic Plot Arrangement upon the Estimate of Error in Field Experiments. J. Agric. Sci., 1931, 21, 191-208.
3. T. EDEN and F. YATES, On the Validity of Fisher's z Test when applied to an Actual Example of Non-normal Data. J. Agric. Sci., 1933, 23, 6-17.
4. R. A. FISHER and F. YATES, The 6×6 Latin Squares. Proc. Lond. Math. Soc. (In the press.)
5. L. H. C. TIPPETT, Tracts for Computers. No. XV. Random sampling numbers. Cambridge University Press.

(Received July 22, 1933)

[Extracted from the *Proceedings of the Cambridge Philosophical Society*,
Vol. **xxx**. Pt. **iv**.]

The 6×6 Latin squares. By R. A. FISHER, Sc.D., Gonville and Caius College, and F. YATES, B.A., St John's College.

[Received 9 August, read 29 October 1934.]

1. *Introduction.*

The problem of the enumeration of the different arrangements of n letters in an $n \times n$ Latin square, that is, in a square in which each letter appears once in every row and once in every column, was first discussed by Euler(1). A complete algebraic solution has been given by MacMahon(3) in two forms, both of which involve the action of differential operators on an expanded operand. If MacMahon's algebraic apparatus be actually put into operation, it will be found that different terms are written down, corresponding to all the different ways in which each row of the square could conceivably be filled up, that those arrangements which conflict with the conditions of the Latin square are ultimately obliterated, and those which conform to these conditions survive the final operation and each contribute unity to the result. The manipulation of the algebraic expressions, therefore, is considerably more laborious than the direct enumeration of the possible squares by a systematic and exhaustive series of trials. It is probably this circumstance which has introduced inaccuracies into the numbers of 5×5 and 6×6 Latin squares published in the literature.

The problem of the Latin square has become of practical interest in recent years in connection with the development of an adequate theoretical basis for the design of biological experiments, for as soon as the underlying principles of such design began to be understood, it appeared that the Latin square arrangement was in many respects extremely suitable to a large class of field trials with agricultural crops. The reason for its special suitability lies in its satisfactorily fulfilling two distinct requirements: (1) in equalising more thoroughly than can be done in other ways the fertility of the land on which the different treatments are to be tested, and (2) in allowing, subject to the fixed restrictions of the Latin square, of a random choice among the different possible squares which could be laid down on the same area. This element of randomisation is now recognised to be a necessary condition for the validity of the estimate of error by which the results of the experiment are to be judged, and it is the fact that it is not a particular Latin square but a random selection from an aggregate of possible squares which is required for agricultural practice, that has given a renewed interest to Euler's problem of their enumeration.

For the numbers of reduced squares MacMahon had given

n	1	2	3	4	5
Number	1	1	1	4	52

but on constructing the possible 5×5 squares for agricultural use it was at once found that there were 56 and not 52 possible reduced squares. The corrected number was communicated by one of the authors in 1924 to Professor MacMahon in time to be incorporated in the copies of *Combinatory Analysis* then unsold. As will be seen below, this number, 56, was actually given "*d'après un dénombrement exact*" by Euler in 1781.

The 6×6 squares are too numerous to be enumerated *seriatim* without risk of error. Since this size is eminently suitable for agricultural purposes the method of enumeration given in subsequent sections was developed. In this case also it has been found that the number given by a previous author is incorrect, for Jacob(4), using a systematised method of progressive trial, had arrived at the number 8192, whereas, as will be shown, there are in reality 9408 reduced 6×6 squares.

The enumeration of the 6×6 squares is particularly apposite to Euler's main concern, which was to solve or to demonstrate the insolubility of the problem of constructing a 6×6 Graeco-Latin square (*quarré magique complet*). He uses a method of transformation similar to that which we shall call intramutation, but not possessing the invariant diagonal properties which we shall use, in order to show that the 6×6 Latin squares may be treated in sets such that of each set all or none are eligible as a basis for forming Graeco-Latin squares. Without making an exhaustive enumeration of the different sets, which must be at least as numerous as the 12 sets which may be generated by a transformation more general than Euler's, Euler argued as follows (p. 229):

"De là il est clair, que s'il existoit un seul quarré magique complet de 36 cases on en pourroit déduire plusieurs autres moyennant ces transformations, qui satisferoient également aux conditions du problème. Or ayant examiné un grand nombre de tels quarrés, sans avoir rencontré un seul, il est plus que probable, qu'il n'y en ait aucun; Car le nombre des latins ne sçauroit être si énorme, que la quantité de ceux que j'ai examiné n'en devroit avoir fourni un qui admet des directrices, s'il y en avoit; vû que le cas $n = 2$ et $n = 3$ ne fournit qu'un seul, le cas de $n = 4$ quatre, le cas de $n = 5$ cinquante six, d'après un dénombrement exact, d'où l'on voit que le nombre des variations pour le cas de $n = 6$ ne sçauroit être si prodigieux, que le nombre de 50 ou 60, que je pourrais avoir examiné n'en fut qu'une petite partie."

Had Euler realised that the number of 6×6 Latin squares of reduced form was as high as 9408, and especially that a number of the transformation sets contain less than a hundredth of the total, he would probably not have judged his conclusion *plus que probable*. On this point a rigorous test is now available by examining for possible systems of *directrices* members of the 12 adjugate sets chosen from the 17 examples given in Section 4. This test has been made, and it was found that none of the 12 sets gave any concordant system of *directrices*. It follows, therefore, as Euler confidently predicted, that no 6×6 Graeco-Latin square can exist.

The discussion of the present paper may be contrasted with those of Cayley(2), MacMahon, and Jacob in turning not on the conditions to be satisfied by a permutation by which one line of the square may be transformed into the next, but on the intrinsic symmetry which each solution of the problem of the Latin square presents as a whole. We are therefore concerned with the operations by which a square can be transformed into other squares having the same structural symmetry, whereas the above-mentioned authors have considered the Latin square as a special case of the Latin rectangle.

2. Definitions.

1. *Reduced Latin squares.* A square with the first row and first column in alphabetical order *ABCDEF...* has been named by MacMahon a *reduced Latin square*. The diagonal passing through the intersection of the first row and column of a reduced square will be called the *leading diagonal*. A pair of squares is said to be *conjugate* when one is the mirror image of the other in the leading diagonal. *Self-conjugate squares* are symmetrical about the leading diagonal.

2. *Adjugate squares.* Just as the interchange of the rows and columns of a square will give the conjugate square so the interchange of rows and letters in each element (the letters being regarded as possessing, like the rows, a serial order) or of columns and letters will generate a series of squares which may be spoken of as mutually *adjugate*.

3. *Transformations.* Any permutation of the rows, columns, or letters of a square, among themselves, or combination of such permutations, generates another square (possibly identical with the original square). Any rearrangement of this nature will be called a *transformation*.

4. *Intramutations.* In a reduced Latin square any permutation of all the letters other than *A* may be made, and the rows and columns (excluding the first) then rearranged so as to give another reduced

Latin square by arranging the letters of the first row and first column in the standard order. A transformation of this type will be styled an *intramutation*.

Any square of order n can be transformed in $(n!)^2$ ways (including no change). All these transformations do not necessarily give different squares, but all possible squares of order n can be classified in sets of squares which are derivable from one another by some transformation.

It is easily seen that a sequence of transformations is itself a transformation, and that for each transformation there exists a reciprocal transformation which reverses its effect. From these properties it follows that every square of a transformation set is derivable from every other square of the set by the same number of transformations. Thus if there are s and only s transformations which when applied to a square P give square Q , every square in the set is connected to every other square by s transformations, and to itself by $s - 1$ transformations (excluding no change); and there are consequently $(n!)^2/s$ squares in the set.

The same property holds good also for intramutation sets of reduced Latin squares: if there are t and only t intramutations connecting any pair of reduced squares then they must be members of an intramutation set containing $(n - 1)!/t$ reduced squares.

Each reduced square generates a set of $n!(n - 1)!$ squares, all different, by permutation of all the columns, and all the rows except the first. Only the original square is a reduced square. It is therefore sufficient to enumerate all the reduced squares of the size under consideration.

3. Enumeration of the 6×6 squares.

There does not appear to be any generating process which when applied to a reduced square will generate a fixed number of other reduced squares, all different. The process of intramutation, however, enables the enumeration to be carried out by sets of varying sizes, of which 120 is the largest and most frequent, members of the same set having certain characteristics of the leading diagonal which are unaltered by intramutation. The actual enumeration consists of three stages.

(a) *The exhaustive enumeration of the possible types of leading diagonals.* These are listed for the 6×6 squares in the first and last columns of Table I. For example, diagonals containing two different letters other than A and four A 's can all be derived from diagonals containing four A 's, one B and one C . All diagonals containing these letters are not, however, derivable from one another; for if, for example, B falls in the column headed by C and C in the column headed by B this property is invariant, and intramutations of the other letters, or B and C , will not change it. It will be seen

that any diagonal containing two different letters other than *A* and four *A*'s can be derived from one of the diagonals *ACBAAA*, *ACAAAB*, or *AAAABC*, in which respectively both, one, or neither letter falls in the column headed by the other letter. These three diagonals are therefore taken as examples of the three diagonal types containing two different letters other than *A*.

(b) *The determination of the number of distinct diagonals which can be generated by intramutation from each typical diagonal.* This presents no difficulty. There are, for example, 10 possible diagonals of the type *ACBAAA*, and 60 of the type *ACAAAB*.

(c) *The enumeration by trial of all possible reduced squares having the given typical diagonals.* This task, though it appears considerable, is not really onerous for 6×6 squares. In some cases intramutations which leave the diagonal unchanged may be used to shorten the enumeration.

When these operations have been performed the number of squares is determined. The number of squares having the typical diagonal *ACBAAA*, for example, is 8, and since the number of diagonals derivable from the typical diagonal (including the diagonal itself) is 10, this diagonal contributes 80 squares to the total.

Intramutations which leave the diagonal unaltered may give a different square, and consequently all the squares having a given typical diagonal do not necessarily belong to different intramutation sets. Thus by applying a suitable selection of those intramutations which leave the diagonal *ACBAAA* unchanged to the 8 squares having this diagonal (4 squares and their conjugates) it is found that two squares are connected by intramutation to their conjugates, and the other two each to the conjugate of the other. There are thus four intramutation sets, all of 20 squares; two of these include conjugates, and the other two form a conjugate pair of sets, every square of one set being conjugate to a square of the other. The four sets are thus representable on three cards. The complete classification is set out in Table I. There are in all, as will be seen from the table, 8 self-conjugate sets, 25 sets including conjugates and 39 conjugate pairs of sets.

To obtain a general permutation of letters, intramutation must be supplemented by a change of another type. This consists of bringing any chosen one of the 36 letters to the top left-hand corner by permutation of the rows and columns, at the same time interchanging this letter with *A*. The rows and columns can be rearranged in the appropriate order to give a reduced square, and with this restriction there will be 36 such changes (which we call *change of corner element*). The 36 changes of corner element, together with the $6! 5!$ permutations of rows and columns giving

non-reduced squares, combine with the $5!$ possible intramutations to give the whole $(6!)^3$ permutations of the general transformations.

The determination of the connections between the intramutation sets of the 6×6 squares is fairly easily performed, for when once the 36 diagonals generated by change of corner element have been written down for any one square, and identified with typical diagonals, the number of squares with which the given square can possibly be connected is seen to be very limited, and only a few full transformations are necessary. There cannot be any connection between two squares which give a different set of 36 typical diagonals, so that there are few cases where there is any danger of overlooking a connection. The whole process forms an excellent check on the original enumeration.

These connections are also exhibited in Table I, by means of the Roman numerals. The whole of the 111 intramutation sets are comprised by 22 transformation sets, 10 of which form 5 conjugate pairs. Examples from each of these sets are exhibited in the next section. The pair of conjugate sets with diagonal *ACBAAA*, for instance, belongs to the pair of conjugate transformation sets illustrated in Example XI. The only other two pairs of intramutation sets in this pair of transformation sets are the pairs (containing 60 and 40 squares) with diagonals *ACBBCA* and *ACBBBB*. Each of the pair of transformation sets therefore comprises 120 squares.

The greatest number of reduced squares found in any one set is 1080. If every transformation gave a different square there would be $6^2 \cdot 5! = 4320$ reduced squares in the set. The least number of connections found in sets of 6×6 squares is therefore 4.

The adjugacy of transformation sets, i.e. the connections introduced by the interchange of rows with columns or either with letters, is easily established and will be discussed in the next section.

The grand total of all reduced 6×6 Latin squares is 9408, and therefore the total number of 6×6 squares is $9408 \cdot 6!5! = 812,851,200$. Jacob(4) obtains 8192 as the number of reduced 6×6 squares. He based his enumeration on the enumeration by trial of all reduced squares having given typical second rows. Since this grouping cuts across the intramutation grouping on which our enumeration is based it is not possible to locate the discrepancies without considerable labour. It is, however, certain that the true number is not less than that of those which we have enumerated, even if we consider the remote possibility that any set has been omitted. On this latter point the reader will, we think, find little difficulty in verifying that the typical diagonals have been completely listed.

It is interesting to notice that the number 9408 is 3 times 56^2 , 56 being the number of 5×5 reduced squares. Similarly 56 is

$3\frac{1}{2}$ times 4^2 , 4 being the number of 4×4 reduced squares, and 4 is 4 times 1^2 , 1 being the number of 3×3 reduced squares. This consideration, for what it is worth, suggests that the number of 7×7 reduced squares may be expected to be of the order of two hundred and fifty million.

4. The twenty-two transformation sets.

We now give 17 examples illustrating the 22 transformation sets which have been found.

	I	II
No. of squares:	1080, 1080	(180s + 450 + 450c)
	A B C D E F	A B C D E F
	B C F A D E	B C F E A D
	C F B E A D	C F B A D E
	D E A B F C	D E A B F C
	E A D F C B	E A D F C B
	F D E C B A	F D E C B A
Serial-numbers:	0001-1080	2161-3240
	1081-2160	

Example I stands for a conjugate pair of sets of 1080 reduced squares, and Example II for a set adjugate to this pair, containing 180 self-conjugate pairs, one of which is chosen as our example, together with 450 unsymmetrical squares with their 450 conjugates. This trio of sets comprises 3240 reduced squares, which is the largest number that can be illustrated by a single example.

In respect of the occurrence of self-conjugate squares it is worth noting that the elements of the leading diagonal in any of the self-conjugate members are invariant in the sense of being unchanged by intramutation, or by change of corner element within the elements of the diagonal. The number of self-conjugate squares in any transformation set must therefore be one or more sixths of the number of squares in the set.

III	IV
(540 + 540c)	(540 + 540c)
A B C D E F	A B C D E F
B A F E C D	B A E F C D
C F B A D E	C F B A D E
D C E B F A	D E A B F C
E D A F B C	E D F C B A
F E D C A B	F C D E A B
3241-4320	4321-5400

Examples III and IV each stand for a set of 1080 reduced squares. Both the sets comprise all squares conjugate or adjugate to any of their members, and consequently, since there are no self-conjugate squares in either group, each comprises 540 squares and their conjugates.

Adjugacy can connect only transformation sets of the same number of reduced squares, and might theoretically connect either six, three, two, or one such set. In the case of only two sets these may be a conjugate pair, but not a pair of sets containing conjugates, such as those of Examples III and IV. This can be shown by considering the effects of successive interchanges of rows with columns and rows with letters.

In the case of the 6 × 6 squares, the only possible adjugate sets which include more than one transformation set are those consisting of a conjugate pair of transformation sets and one set including conjugates. Examples I and II, already given, are illustrative of this grouping, and, as will be seen as we proceed, all conjugate pairs of transformation sets form parts of adjugate trios of this nature, though there does not appear to be any reason why this should hold universally for squares of higher orders.

V	VI
540, 540	(90s + 225 + 225c)
A B C D E F	A B C D E F
B A E C F D	B A F E C D
C F B A D E	C F B A D E
D E F B C A	D E A B F C
E D A F B C	E C D F B A
F C D E A B	F D E C A B
5401-5940	6481-7020
5941-6480	

After the five sets containing 1080 squares each, come four sets each containing 540, these comprising an adjugate trio, and a further single set. Example V illustrates the conjugate pair of sets of 540 squares each, and Example VI the set of 540 which is adjugate to this pair. Of this set one-sixth are self-conjugate (as is the example shown) and the remainder consequently constitute 225 conjugate pairs. This trio therefore comprises 1620 reduced squares.

TABLE I.

Possible diagonals (1)	Self-conjugate sets (2)	Number of squares in		Cards (5)	Sets (6)	Squares (7)	Impossible diagonals (8)
		Sets including conjugates (3)	Sets belonging to conjugate pairs (4)				
AAAAAA	6 (XVI)	30 (IV)	6 (XV)	3	4	48	AAAAAB
AAAAAB	60 (VI)	60 (IX)	—	2	2	120	
AAAABC	—	—	60 (V, VIII)	2	4	240	
ACAAAB	—	120 (VII)	—	1	1	120	
ACBAAA	—	20 (XII, XVII)	20 (XI)	3	4	80	AAABBB
AAABBC	—	120 (IX)	—	1	1	120	
ACAABB	—	120 (IV)	—	1	1	120	
AABABC	—	—	120 (I, IV)	2	4	480	
AABACD	—	—	120 (VII)	1	2	240	ADBCAA
AABCD A	—	—	120 (VIII)	1	2	240	
ACBA DA	—	120 (II, VII)	—	2	2	240	
							ACABBB
							AABBBB
							ACBABB
ABBBBB	30 (VI)	30 (IV), 120 (III)	30 (V)	4	5	240	ACBABD
AABBBB	120 (II)	120 (IV)	—	2	2	240	
ACBB CA	60 (II, X, XII, XIII)	120 (II)	60 (I, X, XI)	8	11	720	
ABBB CD	—	—	60 (V, VIII)	2	4	240	
AABCB D	—	—	120 (VIII)	1	2	240	ACBBAD
ACABBB	—	—	120 (I, III)	2	4	480	
AADCB B	—	60 (II, VII)	60 (I)	3	4	240	
ADACBB	—	—	60 (III)	1	2	120	
ACDBAB	—	—	120 (I, V)	2	4	480	ADBCAE
AABCB DE	—	—	120 (IV)	1	2	240	
AABEDC	—	120 (VI, IX)	—	2	2	240	
ACDEBA	—	—	30 (V, XV)	2	4	120	
ACBEDA	—	30 (VI, XVI), 60 (VI, IX)	30 (IV)	5	6	240	ACBCBB
ACBBBB	—	40 (XII, XIV), 120 (II, III)	40 (XI), 120 (I)	6	8	640	
							ADBCBB
							ACBBDB
							ADBBCC
ADBCBC	—	—	120 (I, I, V)	3	6	720	ACBEDB
ACBCBD	—	—	120 (I, II, III, IV, VI)	5	10	1200	
AEB CDB	—	—	120 (V)	1	2	240	
ADBCBE	—	—	120 (I, II, III)	3	6	720	
							ACDEFB
							ACBEFD
29	8	25	39	72	111	9408	18

VII

(270 + 270c)

A	B	C	D	E	F
B	C	D	E	F	A
C	E	A	F	B	D
D	F	B	A	C	E
E	D	F	B	A	C
F	A	E	C	D	B

7021-7560

There remains one set of 540 squares, illustrated by Example VII. This set comprises all squares conjugate or adjugate to any of its members.

VIII

360, 360

A	B	C	D	E	F
B	A	E	F	C	D
C	F	A	E	D	B
D	C	B	A	F	E
E	D	F	C	B	A
F	E	D	B	A	C

7561-7920

7921-8280

IX

(180 + 180c)

A	B	C	D	E	F
B	A	E	F	C	D
C	F	A	B	D	E
D	E	B	A	F	C
E	D	F	C	B	A
F	C	D	E	A	B

8281-8640

The nine sets illustrated so far comprise 7560 squares, leaving only 1848 for the remaining 13 smaller sets. Of these three sets have 360 each, and form an adjugate trio accounting for more than half the remainder. Example VIII represents the conjugate pair of this trio and Example IX the single set of 360, which in this case contains no self-conjugates but 180 conjugate pairs.

X

(60s + 60 + 60c)

(a)

A	B	C	D	E	F
B	C	F	A	D	E
C	F	B	E	A	D
D	A	E	B	F	C
E	D	A	F	C	B
F	E	D	C	B	A

(b)

A	B	C	D	E	F
B	A	D	C	F	E
C	D	F	E	B	A
D	C	E	F	A	B
E	F	B	A	D	C
F	E	A	B	C	D

8641-8820

Only one of our sets, that illustrated in X(a) and X(b), comprises 180 reduced squares, every two of which are therefore connected by 24 distinct transformations. One-third of the squares in this set are self-conjugate, there being two invariant sets of elements each of which may constitute a diagonal of symmetry.

A new feature is introduced in this transformation set in that twelve of the arrangements, such as that illustrated in Example X(b), consist of nine 2×2 squares. These nine 2×2 squares must of course consist of three groups of three squares each, arranged in a 3×3 Latin square, squares of the same group containing the same pair of letters. The squares of any group may be all oriented alike or two may be oriented alike and the third at right angles to them. In the set of squares under discussion the 2×2 squares of the same group are never oriented alike for all three groups. For this reason the squares termed by Euler squares *à double marche* cannot occur in this group.

XI

120, 120

A	B	C	D	E	F
B	C	A	F	D	E
C	A	B	E	F	D
D	F	E	B	A	C
E	D	F	C	B	A
F	E	D	A	C	B

8821-8940

8941-9060

XII

(60s + 30 + 30c)

A	B	C	D	E	F
B	C	A	E	F	D
C	A	B	F	D	E
D	E	F	B	A	C
E	F	D	A	C	B
F	D	E	C	B	A

9061-9180

There are three sets of 120, forming an adjugate trio. The two forming the conjugate pair are shown in Example XI, and the third, which includes 60 symmetrical squares, in Example XII. In this set, therefore, there are three sets of six elements each capable of appearing as diagonals of symmetry. These transformation sets (Examples XI and XII) all comprise arrangements consisting of four 3×3 Latin squares, as is shown in the examples chosen.

XIII

60s

(a)						(b)					
A	B	C	D	E	F	A	B	C	D	E	F
B	C	A	F	D	E	B	C	D	E	F	A
C	A	B	E	F	D	C	D	E	F	A	B
D	F	E	B	A	C	D	E	F	A	B	C
E	D	F	A	C	B	E	F	A	B	C	D
F	E	D	C	B	A	F	A	B	C	D	E

9181-9240

The remaining sets comprise only 228 squares in all. The set of 60, Example XIII, illustrates the possibilities of symmetry about the diagonal in the highest degree, for every element of the square belongs to one or other of six sets, each of which is capable of appearing as a diagonal of symmetry, and consequently every reduced square of the set is self-conjugate. This set is remarkable in that four members consist of nine 2×2 squares (of which one is Euler's square *à double marche*) and six other members of four 3×3 squares (including Euler's *triple marche*), but the properties of this peculiarly simple class of Latin squares may be most easily developed by throwing it into the form shown in Example XIII(b), in which each line is shifted one place from its position in the line above, and all lines at right angles to the leading diagonal contain only a single letter. This is Euler's arrangement *à simple marche*. The whole transformation set comprises what Jacob has termed "complete cycle" squares (if his definition is taken to include, as he implies, the production of the rows in any order by the "complete cycle" operator). Jacob gives a formula for the number of reduced "complete cycle" squares of side n , namely $(n-1)!/\Phi(n)$, where $\Phi(n)$ is the number of integers, including unity, less than n and prime to it.

XIV

(20 + 20c)

A	B	C	D	E	F
B	C	A	E	F	D
C	A	B	F	D	E
D	F	E	B	A	C
E	D	F	C	B	A
F	E	D	A	C	B

9241-9280

There is one set of 40 squares, Example XIV, which comprises 20 squares and their conjugates. Four members of the set consist of four 3×3 squares. This set is interesting in that, like the set represented by Example XIII, it and all adjugate squares formed from its members comprise only a single intramutation set.

XV						XVI					
36, 36						(6s + 15 + 15c)					
A	B	C	D	E	F	A	B	C	D	E	F
B	A	F	E	D	C	B	A	E	C	F	D
C	D	A	B	F	E	C	E	A	F	D	B
D	F	E	A	C	B	D	C	F	A	B	E
E	C	B	F	A	D	E	F	D	B	A	C
F	E	D	C	B	A	F	D	B	E	C	A
9281-9316						9353-9388					
9317-9352											

The three sets of 36 squares, which form the fifth and last adjugate trio, are represented by Examples XV and XVI, the former illustrating the conjugate pair of sets, and the latter the third set of the trio, of which in this case one-sixth are self-conjugate. Although these sets are so small none of them contains squares which can be broken up into 2×2 or 3×3 squares.

XVII					
(10 + 10c)					
A	B	C	D	E	F
B	C	A	F	D	E
C	A	B	E	F	D
D	E	F	A	B	C
E	F	D	C	A	B
F	D	E	B	C	A
9389-9408					

Example XVII represents the smallest set of 20 reduced squares, and concludes our enumeration of the 9408 reduced squares. Like the sets of Examples XIII and XIV it comprises only a single intramutation set, and one-tenth of the members consist of four 3×3 squares.

The classification of 4×4 and 5×5 squares, on lines similar to those which we have employed with the 6×6 squares, may be given

here for purposes of comparison. In the case of the 4×4 squares the 4 reduced squares, all of which are self-conjugate, form two intramutation sets, one containing three squares (the "complete cycle" squares) and the other a single square. Each of these sets, with the corresponding non-reduced squares, constitutes a transformation set. In the case of the 5×5 squares the 56 reduced squares form six intramutation sets, of which five belong to a transformation set containing the 25 unsymmetrical reduced squares and their conjugates, and the other forms a transformation set containing the 6 self-conjugate reduced squares, the set of "complete cycle" 5×5 squares. These are tabulated in Table II, an example from each transformation set being given. It is clear that each transformation set of both 4×4 and 5×5 squares must contain all squares adjugate to any of its members.

TABLE II.

Intramutation sets for 5×5 squares.

Diagonals	Number of squares in			Sets	Squares
	Self-conjugate sets	Sets including conjugates	Sets belonging to conjugate pairs		
AAAAA	—	2 (I)	—	1	2
AABBB	—	8 (I)	—	1	8
AAECD	—	—	8 (I)	2	16
ACBBB	—	24 (I)	—	1	24
AEB CD	6 (II)	—	—	1	6
5	1	3	1	6	56

I	II
(25 + 25c)	6s
A B C D E	A B C D E
B A D E C	B C D E A
C E A B D	C D E A B
D C E A B	D E A B C
E D B C A	E A B C D

5. Conclusions.

1. It has been shown that the number of reduced 6×6 Latin squares can be enumerated without extravagant labour. This is done by means of the special type of transformation which we term

intramutation, and is made possible through the existence of properties of the leading diagonal which are invariant under such transformations. The number of reduced squares is thus enumerated for the most part in sets or pairs of sets of 120, i.e. $(n-1)!$; only a minority of the squares belong to smaller sets. If the same method were to be applied to 7×7 squares, supposing there to be about 250,000,000 of these, we must anticipate nearly 200,000 sets or pairs of sets, since the greatest number in any such pair will be $2.6!$ or 1440.

2. On the other hand, using the sets generated by a general transformation involving independent permutations of rows, columns, and letters, the aggregate of 6×6 squares has been shown to be easily derivable from only 17 examples, representing only 12 distinct types of squares. It is not easy to suppose that any similar grouping could reduce the number of typical 7×7 squares below about 10,000, so that their enumeration would, by any means at present available, be exceedingly tedious.

3. The number of reduced 6×6 Latin squares given by Jacob, namely 8192, is too small, for our complete enumeration gives 9408 reduced squares. This happens rather oddly to be 168 times 56, the number of reduced 5×5 squares, or three times the square of that number.

4. Euler's conclusion that no Graeco-Latin 6×6 square exists is easily verified from the 12 types of 6×6 Latin squares exemplified in this paper.

REFERENCES

- (1) L. EULER, "Recherches sur une nouvelle espèce de quarrés magiques", *Verh. v. h. Zeeuwisch Genootsch. der Wetensch., Vlissingen*, 9 (1782), 85-239.
- (2) A. CAYLEY, "On Latin squares", *Mess. of Math.* 19 (1890), 135-137.
- (3) P. A. MACMAHON, *Combinatory Analysis* (Cambridge, 1915, 1916).
- (4) S. M. JACOB, "The enumeration of the Latin rectangle of depth three by means of a formula of reduction, with other theorems relating to non-clashing substitutions and Latin squares", *Proc. Lond. Math. Soc.* (2), 31 (1930), 329-354.

THE ANALYSIS OF MULTIPLE CLASSIFICATIONS WITH UNEQUAL NUMBERS IN THE DIFFERENT CLASSES

By F. YATES, *Rothamsted Experimental Station, England*

A type of problem which frequently confronts the statistician is the analysis of data which can be classified simultaneously in two or more different ways, as for example, the analysis of the incidence of disease in different factories, where the factories might be classified according to type of work and also according to geographical position. The statistical procedure appropriate to the case where the numbers in the various sub-classes are equal is specially simple, and has been very fully developed in connection with replicated field trials in agriculture. The procedure is a special case of the method known as the analysis of variance, which was first introduced by R. A. Fisher.

When analyzing tables in which the numbers of the various sub-classes are unequal the procedure appropriate to equal numbers requires considerable modification. A. E. Brandt¹ in a paper in this JOURNAL has set out a method of analyzing a $2 \times s$ table. The present paper considers the more general case of a $p \times q$ table, and suggests certain corrections which appear to be necessary in Brandt's methods. The matter has already been discussed in a recent paper² but only somewhat incidentally in connection with rather different problems of experimental design. It is proposed here to give a rather more detailed account of the methods of analysis and the logical principles underlying them.

ESTIMATION AND TESTS OF SIGNIFICANCE

In the analysis of any given body of data the practical statistician is usually concerned with the two fundamentally different statistical problems of estimation and tests of significance. The object of estimation is to obtain the best value available from the data for the magnitude of an effect assumed to exist, whereas the object of tests of significance is to decide whether there is any evidence that the effect exists at all. Both these problems necessitate certain assumptions before we can proceed to their solution.

In estimation the data are assumed to be a random sample from a

¹ A. E. Brandt, "The Analysis of Variance in a $2 \times s$ table with Disproportionate Frequencies," this JOURNAL, 1933, 28, 164.

² F. Yates, "The Principles of Orthogonality and Confounding in Replicated Experiments," J. Agric. Sci., 1933, 23, 108.

population of some given mathematical type, but with certain constants undetermined. For example, in measuring the height of a mountain by observing its angle of elevation from a place a known distance away various values might be obtained. The common practice is to take the mean of all the values as the best estimate of the height. Yet the mean is only the best estimate if the observed values can be regarded as a sample of a normally distributed population of values (or of certain other special types of distribution). We may regard this as a reasonable assumption, partly in view of our knowledge of the sources of the errors to which the observations are subject, partly by previous experience with similar observations, partly by examination of the observations themselves. But the assumption (at least of approximate normality) must be made, for there are other types of distribution in which the observations might conceivably be distributed in which the mean is by no means the best estimate, some in fact in which the mean is a very poor estimate indeed. This is implicitly recognized by the surveyor in that he usually reserves himself the right to reject widely discordant observations.

Tests of significance depend on a similar assumption as to the existence of an infinite population of values giving rise to the particular values observed, but here the question propounded is somewhat different, being of the form: What is the probability that an estimate from a set of values can be regarded as a member of some population of estimates, or alternatively what is the probability that two or more estimates from different sets of values can be regarded as belonging to the same population of estimates? If this probability is small it is taken as evidence that the two sets of values are measures of different quantities. In all cases the form of the population of estimates is determined, once the form of the population of values is assumed (though the available methods of mathematical analysis may not be powerful enough to define it) but the observations themselves may be utilized to determine some or all of the parameters. Consider, for example, estimates of the height of children of a certain age made by measuring the height of two groups of children, one from well-to-do and the other from poor parents. Assuming that both sets of heights are samples from the same normally distributed population of heights it is possible to use the observations themselves to evaluate the probability of obtaining two sets of heights with means differing more widely than the means of the two observed sets differ. If such a probability is low (say less than 1 in 20 or 1 in 100) the two estimates of mean height are said to be significantly different, and if adequate precautions have been taken to eliminate or equalize other disturbing

factors, this may be regarded as evidence that there is in fact a real difference attributable to causes associated with parentage.

In the case of multiple classifications with unequal class numbers, estimates and tests of significance vary according as certain assumptions are or are not made. We will here confine our attention to the discussion of a double (or two-way) classification, except for a brief note on multiple two-fold classifications. The extension to more complex classifications involves no new principle.

Assuming that each value can be classified in one of p classes representing effects A and simultaneously in one of q classes representing effects B , there will be pq sub-classes, each of which may contain several, one, or no values. The numbers in each sub-class (the class numbers, n_{11} , etc.) may be set out in a two-way table, Table I, the marginal totals being represented by $N_{1.}$, etc., $N_{.1}$, etc., and the general total by N .

TABLE I
CLASS NUMBERS

	A_1	A_2	A_p	Total
B_1	n_{11}	n_{21}	n_{p1}	$N_{.1}$
B_2	n_{12}	n_{22}	n_{p2}	$N_{.2}$
.
B_q	n_{1q}	n_{2q}	n_{pq}	$N_{.q}$
Total	$N_{1.}$	$N_{2.}$	$N_{p.}$	N

If y be taken as the general symbol for an observed value the values in the sub-class $A_1 B_1$ may be represented as y_{111} , y_{112} , etc. and the sub-class mean as \bar{y}_{11} . The mean of all the A_1 sub-class means may be written as $\bar{y}_{1.}$, and the mean of all the sub-class means as \bar{y} . Similarly the sum of all the values in the sub-class $A_1 B_1$ may be written Sy_{11} , the sum of all the A_1 sub-class sums as $SSy_{1.}$ and the grand total SSy .

The values y will be assumed to be normally distributed with the same variance about hypothetical sub-class means.

An estimate of this variance is immediately available from the differences of the individual values from their own sub-class means, the estimates from all the sub-classes being pooled in the ordinary manner of the analysis of variance with unequal classes (outlined in the next section). The difference between any two sub-class means can then be tested by means of the t test, and the difference between any pair of means of sub-class means, weighted or unweighted, may be tested in a similar manner. It should be borne in mind that such tests will frequently be adequate to decide on the significance or otherwise of the

effects under consideration, and that the more elaborate tests about to be described will not then be necessary. But whether tests of significance involving several degrees of freedom are undertaken or not, the various possible estimates of the different effects should be clearly distinguished.

The pq hypothetical sub-class means, if known, might be combined in various ways corresponding to real physical effects. In particular, in a two-way classification the differences of the means of all sets of hypothetical sub-class means having the same A may be taken as representing (or more strictly as defining) the average A effects. The average B effects may be similarly defined.

The observed sub-class means are efficient estimates of the hypothetical sub-class means, and in the absence of any further assumptions the efficient estimates of the average A effects are obtained by taking the means of the observed sub-class means for like A over all B sub-classes. In the above notation these means are $\bar{y}_1, \bar{y}_2, \text{etc.}$

It may happen, however, that the phenomena we are investigating are such that the A and B effects are additive, so that the hypothetical sub-class means are of the form

$$\mu + \alpha_r + \beta_s; r = 1, 2, \dots p; s = 1, 2, \dots q;$$

where μ may be called the hypothetical general mean and the α 's and β 's the hypothetical deviations due to the treatments, these being subject to the relations

$$\begin{aligned}\alpha_1 + \alpha_2 + \dots + \alpha_p &= 0, \\ \beta_1 + \beta_2 + \dots + \beta_q &= 0.\end{aligned}$$

In this case the differences of $\bar{y}_1, \bar{y}_2, \text{etc.}$, though still unbiased estimates of the average A effects (which now correspond to the differences of $\alpha_1, \alpha_2, \text{etc.}$), are no longer efficient estimates. Efficient estimates are given by the method of maximum likelihood, which is here equivalent to the method of least squares.

In certain cases it is possible to say definitely, from the nature of the experimental material, that the additive law holds, but more frequently the additive law must be regarded as a possible descriptive law, which is essentially an approximation to some more complex law. We may, however, be prepared to assume that the departures from the additive law are negligible on the ground that they are small in comparison with the errors of the data.

Departures from the additive law are generally termed interactions. The significance or otherwise of the interactions as a whole may be tested by finding the residual variance between sub-class means

after fitting constants representing additive effects and comparing with the variance within classes. This, of course, does not provide any absolute criterion as to whether the assumption of negligible interactions is justified, but it is a useful indication.

Tests of significance of the average A effects are different according as the interactions are assumed non-existent, or not. If the assumption is made the fitted constants must be tested for significance, in the ordinary manner of the method of least squares; if the assumption is not made then the method of weighted squares of means described in the next section can be used.

METHOD OF WEIGHTED SQUARES OF MEANS

The appropriate procedure for the analysis of variance of a single classification with unequal numbers in the various classes has been described by Fisher¹, § 44. Suppose that the class totals and means, and numbers of observations in each class are given by Table II. The

TABLE II

Class	A_1	A_2	A_p	All
Class total.....	Sy_1	Sy_2	Sy_p	Sy
Class mean.....	\bar{y}_1	\bar{y}_2	\bar{y}_p	\bar{y}
Number of observations.....	n_1	n_2	n_p	N

total sum of squares is $SS(y - \bar{y})^2$, corresponding to $N - 1$ degrees of freedom, which can be split up into $p - 1$ degrees of freedom involving comparisons between the class means, and $N - p$ degrees of freedom involving comparisons within each class only. Class A_1 contributes $n_1 - 1$ degrees of freedom to the latter, and the corresponding sum of squares is clearly $S(y_1 - \bar{y}_1)^2$, the total sum of squares for the $N - p$ degrees of freedom within classes being

$$S(y_1 - \bar{y}_1)^2 + S(y_2 - \bar{y}_2)^2 + \dots$$

In virtue of the identity

$$SS(y - \bar{y})^2 = S(y_1 - \bar{y}_1)^2 + S(y_2 - \bar{y}_2)^2 + \dots + n_1(\bar{y}_1 - \bar{y})^2 + n_2(\bar{y}_2 - \bar{y})^2 + \dots,$$

the remaining portion of the sum of squares for the $p - 1$ degrees of freedom can be computed from the formula

$$\begin{aligned} Q &= n_1(\bar{y}_1 - \bar{y})^2 + n_2(\bar{y}_2 - \bar{y})^2 + \dots \\ &= n_1\bar{y}_1^2 + n_2\bar{y}_2^2 + \dots - N\bar{y}^2, \end{aligned}$$

¹ R. A. Fisher, *Statistical Methods for Research Workers*, 1925, Edinburgh: Oliver and Boyd. (4th Edition, 1932.)

which is equivalent to the last set of terms of the identity. When n_1, n_2, \dots are all equal this reduces to the ordinary form used in the analysis of variance with equal numbers in the different classes.

The expression Q , when divided by the number of degrees of freedom, $p-1$, provides an efficient estimate from the differences of the class means of the variance of the individual observations. It will hold equally when $\bar{y}_1, \bar{y}_2, \dots$ are any numbers distributed about some (unknown) mean with variances $\frac{\sigma^2}{n_1}, \frac{\sigma^2}{n_2}, \dots, \sigma^2$ being also unknown

but n_1, n_2, \dots known. We are thus led to the more general case where an estimate of a variance is required from differences of a set of numbers whose variances are known fractions of that variance. From the above expression for Q this is seen to be the weighted sum of the squares of the deviations from the weighted mean of the numbers, divided by $p-1$, the weights being equal to the reciprocals of the known fractions. If the numbers are u_1, u_2, \dots and their variances $\frac{1}{w_1}, \frac{1}{w_2}, \dots$ of the general variance σ^2 , the estimate s^2 of σ^2 is given by

$$Q = (p-1)s^2 = w_1(u_1 - \bar{u})^2 + w_2(u_2 - \bar{u})^2 + \dots \\ = w_1u_1^2 + w_2u_2^2 + \dots - (w_1 + w_2 + \dots)\bar{u}^2, \quad (A)$$

$$\text{where } \bar{u} = \frac{w_1u_1 + w_2u_2 + \dots}{w_1 + w_2 + \dots}.$$

This result is immediately applicable to the analysis of multiple classifications. The variances of the marginal means of the sub-class means are known fractions of the variance of a single observed value, the variance of \bar{y}_1 . (Table I) being $\frac{1}{q^2} \left(\frac{1}{n_{11}} + \frac{1}{n_{12}} + \dots \right) \sigma^2$, etc.

Hence the efficient estimate of the variance from the A means of the sub-class means is given by the substitutions

$$\frac{1}{w_1} = \frac{1}{q^2} \left(\frac{1}{n_{11}} + \frac{1}{n_{12}} + \dots \right), \text{ etc.,} \\ u_1 = \bar{y}_1, \text{ etc.,}$$

in the above formula for Q , equation (A).

This estimate of the variance may be compared with the estimate of variance from the variation within sub-classes by means of the z test. The latter estimate is obtained by the methods described above appropriate to a single classification, regarding each sub-class as an independent class. It will be based on $N-pq$ degrees of freedom.

The estimate of variance from the A means of the sub-class means is

based on $p-1$ degrees of freedom which are independent of (orthogonal to) the $N-pq$ degrees of freedom within classes, but they will be the appropriate set of degrees of freedom for testing the A effects if, and only if, they are composed of a set of single degrees of freedom which between them contain all the information on the A effects.

This will be the case unless the interactions are known not to exist (or we are prepared to assume their non-existence). In the latter event the method of weighted squares of means can only be regarded as an approximation to the rigorous method of least squares. The approximation will become less close as the class numbers become more unequal.

The method of weighted squares of means also has additional interest in a $2 \times s$ table, in that it furnishes a method for computing the sum of squares appropriate to the efficient test of interactions. These sums of squares can be further used to construct the sums of squares required to test the main effects on the assumption of negligible interactions, or as a check on them.

We will now apply the method to the data given by Brandt in the paper already referred to. These data are reproduced in Table III (with the correction of one class number).

TABLE III

Breed	Female		Male		Total	
	Number	Log of per cent bacon	Number	Log of per cent bacon	Number	Log of per cent bacon
Hampshire.....	33	66.55	89	181.04	122	247.59
Duroc Jersey.....	51	98.89	141	281.43	192	380.12
Tamworth.....	13	25.90	17	34.20	30	60.10
Yorkshire.....	4	7.62	9	17.58	13	25.20
Berkshire.....	8	14.64	4	8.20	12	22.84
Poland China.....	15	28.11	32	64.42	47	92.53
Chester White.....	35	66.90	47	90.52	82	157.42
All others.....	12	23.32	23	46.70	35	70.02
Total.....	171	331.73	362	724.09	533	1,055.82

The table of sub-class means is given in Table IV. They are given to six decimal places in order that the sum of squares between classes may be computed by the products of means and totals (using a working mean of 1.9).

The relative weights of the means of the sub-class means are obtained from the reciprocals of the sub-class numbers, Table V. That for Hampshire, for instance, is the reciprocal of $\frac{1}{4} \left(\frac{1}{33} + \frac{1}{89} \right)$ or 96.29. These weights are given in the last column of Table V.

TABLE IV

Breed	Female	Male	Unweighted mean	Difference
Hampshire	2.016667	2.084158	2.025412	0.017491
Duroc Jersey	1.935099	1.995958	1.965528	0.060859
Tamworth	1.992307	2.011765	2.002036	0.019458
Yorkshire	1.905000	1.953333	1.929167	0.048333
Berkshire	1.830000	2.050000	1.940000	0.220000
Poland China	1.874000	2.013125	1.943562	0.139125
Chester White	1.911429	1.925958	1.918694	0.014529
All others	1.943333	2.030434	1.986884	0.087101
Unweighted mean	1.925979	2.001841	1.963910	0.075862

TABLE V
RECIPROCAL OF SUB-CLASS NUMBERS

Breed	Female	Male	Total	Weight of mean
Hampshire	0.03030	0.01124	0.04154	96.29
Duroc Jersey	0.01961	0.00709	0.02670	149.81
Tamworth	0.07892	0.05882	0.13574	29.47
Yorkshire	0.25000	0.11111	0.36111	11.08
Berkshire	0.12500	0.25000	0.37500	10.67
Poland China	0.06667	0.03125	0.09792	40.85
Chester White	0.02857	0.02128	0.04985	80.24
All others	0.08333	0.04348	0.12681	31.54
Total	0.68040	0.53427	1.21467	449.95

The primary analysis of variance within and between classes is given in Table VI. (The total sum of squares is taken from Brandt's paper.)

TABLE VI

	Degrees of freedom	Sums of squares	Mean square	s
Between classes	15	1.2715	0.0848	0.696
Within classes	517	11.7427	0.0227	
Total	532	13.0142		

The effects of classification are clearly significant. On examination of Table IV it is apparent that sex has produced an effect, the males of all eight breeds giving higher values than the females. It is also apparent, without further analysis, that there are significant differences between the breeds. The estimate of the standard error of the Hampshire mean, 2.0254, is $\sqrt{(0.0227/96.29)}$ or 0.0154, and that of the Chester White mean, 1.9187, is 0.0168. The difference of the means, 0.1067, is more than $4\frac{1}{2}$ times its standard error $\sqrt{(0.0154^2 + 0.0168^2)}$ or 0.0228. The significance is undoubted, even allowing for the fact that we have selected the greatest difference. We will, however,

work out the sum of squares and mean square attributable to the whole 7 degrees of freedom by the method of weighted squares of means, both as an illustration of procedure, and in order later to make a comparison with the values obtained when interactions are assumed non-existent.

The sum of squares attributable to the whole 7 degrees of freedom is computed as follows. The sum of the weights is 449.95, and the weighted mean of the means is therefore

$$\bar{u} = (2.025412 \times 96.29 + 1.965528 \times 149.81 + \dots) / 449.95 \\ = 1.970385.$$

Hence the appropriate sum of squares, using the working mean of 1.9, is given by

$$Q = 0.125412^2 \times 96.29 + 0.065528^2 \times 149.81 + \dots - 0.070385^2 \times 449.95 \\ = 0.6056.$$

It is important, when squaring means instead of totals, to employ a working mean somewhere near the true mean, as otherwise small inaccuracies in the means due to rounding off will seriously affect the result.

The corresponding mean square is 0.0865 (7 degrees of freedom) which when compared with the mean square within classes is quite clearly significant.

The direct effect of sex is measured by the difference 0.075862, and the significance of the difference can be tested immediately by means of the *t* test. Referring to the table of reciprocals of the sub-class numbers, the variance of the mean of the female sub-class means is seen to be $0.68040\sigma^2/8^2$, and the corresponding variance for the males is $0.53427\sigma^2/8^2$. The estimate of the standard error of the difference of these means is therefore $\frac{1}{8}\sqrt{(1.21467 \times 0.0227)}$ or 0.0207, so that *t* is 3.66 (517 degrees of freedom) and the difference is clearly significant.

This test can, if desired, be thrown in analysis of variance form, the relevant mean square being the square of the difference divided by the ratio of its variance to σ^2 , i.e. $0.075862^2.8^2/1.21467$, or 0.3032.

It remains to test whether there is any interaction. In the absence of any interaction the variation between the differences of the male and female sub-class means given in Table IV will be wholly ascribable to error, and in any case the differences of these differences provide efficient estimates of the interactions when they exist. The method of weighted squares of means may therefore be applied to these differences, and will provide an efficient test of the significance of the interactions. The computation proceeds as before, with the exception that

the weights are one-quarter of those shown in Table V. The value obtained for the weighted mean of the differences is 0.053014, and for the sum of squares (7 degrees of freedom) is 0.2299, giving a *z* of 0.185, whereas the 5 per cent point is about 0.35. The interactions, therefore, are not significant.

If the *t* test were applied to the difference between the greatest and least of the differences, the difference would be judged significant. Such a test is not valid because the greatest and least values have been chosen. The method of weighted squares of means takes into account the variation of all the differences instead of only a pair of them.

TABLE VII
ANALYSIS OF VARIANCE—INTERACTIONS NOT ASSUMED NON-EXISTENT

	Degrees of freedom	Sums of squares	Mean squares
Sex.....	1	0.3032	0.3032
Breed.....	7	0.6056	0.0865
Interactions.....	7	0.2299	0.0328

The results are given in Table VII. It should be noted that the sums of squares obtained for breed, sex, and interactions do not total to the sum of squares for the 15 degrees of freedom between sub-classes. The additive property only holds when the degrees of freedom appropriate to the testing of the various effects are mutually independent (orthogonal).

METHOD OF FITTING CONSTANTS

It was pointed out in the second section that the assumption that interactions are non-existent is equivalent to the assumption that the hypothetical sub-class means are additive functions of the constants μ, α, β . This is the assumption which Brandt implicitly makes in his discussion of the analysis of a $2 \times s$ table, for he uses the fact that the marginal totals of a table constructed from the estimates of the constants must be identical with the actual marginal means.

In the general $p \times q$ table the ordinary procedure of the method of least squares gives the following equations for m, a_r, b_s , the estimates of μ, α_r, β_s .

Leading
term

$$\begin{array}{llll} m_1 & Nm + N_{1.}a_1 + N_{2.}a_2 + \dots + N_{.1}b_1 + N_{.2}b_2 + \dots & = & SSy \\ a_1 & N_{1.}m + N_{1.}a_1 & + n_{11}b_1 + n_{12}b_2 + \dots & = SSy_{1.} \\ a_2 & N_{2.}m & + N_{2.}a_2 & + n_{21}b_1 + n_{22}b_2 + \dots = SSy_{2.} \\ & \cdot & \cdot & \cdot \end{array}$$

$$\begin{array}{llll}
 b_1 & N_{.1}m + n_{11}a_1 + n_{21}a_2 + \dots + N_{.1}b_1 & = SSy_{.1} \\
 b_2 & N_{.2}m + n_{12}a_1 + n_{22}a_2 + \dots & + N_{.2}b_2 & = SSy_{.2} \\
 & \cdot & \cdot & \cdot
 \end{array}$$

The rule of formation is as follows. Write, or imagine written, N equations for the N observed values of the form

$$y_{rst} = \mu + \alpha_r + \beta_s + x_{rst}, \quad (B)$$

where x_{rst} is an error term. To form the equation in which m is the leading term, sum the squares of the coefficients of μ in these N equations and the products of these coefficients with the corresponding coefficients of $\alpha_1, \alpha_2, \dots, \beta_1, \beta_2, \dots$ in turn. In this case the coefficient for μ is always unity so the first sum is N , the coefficient of α_1 is unity in the N_1 equations for the observations in all A_1 classes, and zero elsewhere, so that the sum of the products is $N_{.1}$, and so on. These sums give the coefficients of $m, a_1, a_2, \dots, b_1, b_2, \dots$ in the first equation. The numerical term is given by the sum of the products of each observed value with the coefficient of μ in the corresponding equation (B), and thus for the first equation by the sum of all the values. The other equations are formed in the same manner. The whole set of equations gives the values of μ , the α 's and the β 's which make the sum of the squares of the error terms x_{rst} a minimum.

Only $p+q-1$ of the above $p+q+1$ equations are independent, the equation with leading term m being the same as the sums of all the equations with an a as leading term, and of all the equations with a b as leading term.

The solution is best effected by eliminating the b 's or the a 's (whichever are the more numerous). From the b_1 equation

$$N_{.1}b_1 = SSy_{.1} - N_{.1}m - n_{11}a_1 - n_{21}a_2 - \dots \quad (C)$$

Using this and the corresponding values for the other b 's we obtain on substitution in the a equations and some simplification

$$\begin{aligned}
 & \left(N_{.1} - \frac{n_{11}^2}{N_{.1}} - \frac{n_{12}^2}{N_{.2}} - \dots \right) a_1 - \left(\frac{n_{11}n_{21}}{N_{.1}} + \frac{n_{12}n_{22}}{N_{.2}} + \frac{n_{13}n_{23}}{N_{.3}} + \dots \right) a_2 - \dots \\
 & = SSy_{.1} - \frac{n_{11}}{N_{.1}} SSy_{.1} - \frac{n_{12}}{N_{.2}} SSy_{.2} - \frac{n_{13}}{N_{.3}} SSy_{.3} - \dots \quad (D)
 \end{aligned}$$

The a equations are now in the most convenient form for solution, a_p being eliminated by means of the identity $a_1 + a_2 + \dots + a_p = 0$. Only $p-1$ of the equations (D) are independent, the p^{th} being derivable from the sum of all the others. This provides a check. The values

of $m+b_1$, $m+b_2$, . . . can then be derived from (C) by substitution and finally m determined from the identity $b_1+b_2+\dots+b_q=0$.

The formation of the a equations is facilitated by the construction

of a table of the quantities $\frac{n_{11}}{N_{\cdot 1}}, \frac{n_{12}}{N_{\cdot 2}}, \dots, \frac{n_{21}}{N_{\cdot 1}}, \dots$, etc. The numer-

ical factors of the a terms can then be obtained as the straight sums of products of numbers in this table and the table of sub-class numbers.

After the values of the a 's, b 's and m have been obtained the reduction in the sum of squares due to fitting the constants can be calculated as the sum of the products of each constant with the numerical term of the equation of which it is the leading term. This includes the ordinary correction for the mean. Symbolically

$$SSy^2 - SS(y - Y)^2 = mSSy + a_1 SSy_1 + \dots + b_1 SSy_1 + \dots$$

Deducting this, less the correction for the mean, from the sum of squares between classes, we obtain the residual sum of squares between classes corresponding to $(p-1)(q-1)$ degrees of freedom, which is the appropriate sum of squares for testing the significance of the interactions.

If there are only two B classes and $\frac{1}{2}b$ is substituted for b_2 (or $-b_1$), b will be the efficient estimate of the difference of the two B effects. Rewriting equation (D) in terms of b_1 and b_2 we obtain after a little simplification

$$\left(\frac{n_{11}n_{12}}{n_{11}+n_{12}} + \frac{n_{21}n_{22}}{n_{21}+n_{22}} + \dots \right) b = \frac{n_{11}n_{12}}{n_{11}+n_{12}} (\bar{y}_{11} - \bar{y}_{12}) + \frac{n_{21}n_{22}}{n_{21}+n_{22}} (\bar{y}_{21} - \bar{y}_{22}) + \dots$$

In other words b is simply the weighted mean of the differences of the pairs of A sub-class means, and is identical with Brandt's b . The above equation corresponds to his equation (6).

The a 's and m are given by the equations

$$m + a_1 = \frac{1}{n_{11} + n_{12}} \left[SSy_1 + \frac{1}{2} (n_{11} - n_{12}) b \right],$$

etc. These equations give the efficient estimates of the A effects. They can be deduced from Brandt's equations (1) to (4) by the substitution of $m + a_1 - \frac{1}{2}b$ for x , etc.

In the example on pigs already analyzed the values of the a 's and b were found to be

$$\begin{array}{ll}
m+a_1=2.017259 & m+a_5=1.912169 \\
m+a_2=1.967367 & m+a_6=1.959136 \\
m+a_3=1.999799 & m+a_7=1.915877 \\
m+a_4=1.928267 & m+a_8=1.992241 \\
b=+0.053014
\end{array}$$

The discrepancy with Brandt's value for b appears to be due to a computational error. It should be noticed that b has already been determined in computing the interaction sum of squares in the analysis by weighted squares of means.

The reduction in the sum of squares due to the fitting of the constants, excluding the mean, is

$$\begin{aligned}
& 2.017259 \times 247.59 + 1.967367 \times 380.12 + \dots \\
& + \frac{1}{2} 0.053014 (724.09 - 331.73) - (1055.82)^2 / 533.
\end{aligned}$$

In order to avoid the necessity of using an excessive number of decimal places it is better to employ a working mean of 1.9, as before, when the reduction in the sum of squares becomes

$$\begin{aligned}
& 0.117259 \times 15.79 + 0.067367 \times 15.32 + \dots + \frac{1}{2} 0.053014 (36.29 - 6.83) \\
& - (43.12)^2 / 533 = 1.04146.
\end{aligned}$$

It remains to determine the appropriate test of significance for the sex and treatment effects separately. In these tests we differ from Brandt, who deduces formulae from those applicable to equal class numbers. These formulae do not appear to be correct, except that for the interaction sum of squares. The disagreement of his value for the interaction sum of squares in the numerical example with the one given below appears to be due to a misinterpretation of the formula. The computation is also at fault in that the additive property of the various sums of squares is assumed; this does not hold.

The general rule applicable to any groups of fitted constants is to find the part of the sum of squares accounted for by fitting all the constants and deduct from it the part of the sum of squares accounted for by fitting all the constants except those to be tested. In the case of a double classification there is no difficulty in computing the latter sum of squares, even when each classification contains more than two classes. The sum of squares removed by the b constants alone, for instance, is the sum of squares obtained from the B marginal totals, $SSy_{\cdot 1}$, etc., it being remembered that these totals include unequal numbers of observations.

The results in the case of the example already analyzed are given in Table VIII. Using Table III, the sum of squares for breed alone is given by

$$\frac{1}{122} (247.59)^2 + \frac{1}{192} (380.12)^2 + \dots - \frac{1}{533} (1055.82)^2.$$

The sum of squares for sex alone can be computed in the same manner, or by the simpler expression

$$\frac{(171 \times 724.09 - 362 \times 331.73)^2}{171 \times 362 \times 533}.$$

TABLE VIII

	Degrees of freedom	Sums of squares	Mean square
<i>Test for breed:</i>			
Sex alone.....	1	0.4224	
Breed.....	7	0.6191	0.0885
Sex and breed (constants).....	8	1.0415	
<i>Test for sex:</i>			
Breed alone.....	7	0.7253	
Sex.....	1	0.3162	0.3162
Sex and breed (constants).....	8	1.0415	
Interactions.....	7	0.2300	0.0329
Between classes.....	15	1.2715	

Comparing this with Table VII it will be seen that there is little material difference between the mean squares obtained for breed and sex by the two methods. The interaction mean square is the same by both methods, as indeed it should be, being based on the same assumptions. The two methods of computation provide a valuable check. The sum of squares due to sex can also be obtained directly. It is the square of b multiplied by its relative weight, 112.49.

PROPORTIONATE CLASS NUMBERS

If the class numbers may be represented as the product of two numbers, one, h , depending on the A classification and the other, k , on the B classification, so that $n_{rk} = h_r k_s$, they are styled by Brandt proportionate, since the relation $n_{rt}/n_{ru} = n_{st}/n_{su}$ holds generally.

In this case means of the A marginal totals of the table of sub-class totals are efficient estimates of A effects averaged over any class numbers in the B classification *proportional to the actual class numbers*, whether B effects or interactions exist or not. These totals will not contain equal numbers of observations, but the sum of squares appropriate to testing the A effects may be computed by the method already described for a single classification with unequal numbers in the various classes.

The B effects may be estimated and tested in a like manner, and finally the interactions may be tested for significance by deducting

the two sums of squares already obtained from the total sum of squares between classes, since in this case the three sums of squares, for *A* effects, *B* effects, and interactions respectively, are additive.

ANALYSIS OF A $2 \times 2 \times 2 \times \dots$ TABLE

On the assumption of negligible interactions the efficient estimate of the sex effect in the $2 \times s$ table was seen to be the weighted mean of the *s* differences of the pairs of sub-class means for the *s* treatments. All the main effects of a $2 \times 2 \times 2 \times \dots$ table can be estimated and tested by such weighted means, the *t* test being used. If the interactions are not assumed negligible then the unweighted means of the differences must be taken. The individual interactions can be tested by unweighted means in the same manner.

APPROXIMATE METHOD OF ANALYSIS

The variance of any sub-class mean is σ^2/n_r . If the differences between the various class numbers are ignored, all the sub-class means being assumed to have a variance equal to the mean of all the true variances, the methods appropriate to the case of equal class numbers may be employed. This approximation is only useful when the class numbers do not differ very greatly.

Table IX shows the results of applying this method to the pig data. The mean variance of the sub-class means is $0.07592\sigma^2$ (Table V). Analyzing Table IV as if it were an ordinary 2×8 table with a single value in each sub-class, and dividing the sums of squares obtained by 0.07592, Table IX is obtained.

TABLE IX

	Degrees of freedom	Sums of squares	Mean square
Sex.....	1	0.3032	0.3032
Breed.....	7	0.2635	0.0376
Interactions.....	7	0.2387	0.0341
Total.....	15	0.8054	

Comparing these results with those of Table VII and Table VIII, it will be seen that the sum of squares due to sex is the same as that of Table VII, as it should be. The effect of breed, however, is very seriously underestimated. The sub-class numbers are in fact too unequal for this method to give even a tolerable approximation.

SUMMARY

The methods of estimation and tests of significance of the main effects of a two-way table differ according as to whether interactions

are, or are not, assumed non-existent. If the assumption is made the method of fitting constants provides efficient estimates and efficient tests of significance. If it is not made the sub-class means provide efficient estimates, and the method of weighted squares of means efficient tests of significance.

The method of fitting constants also provides efficient tests of the interactions. The method of weighted squares of means provides efficient tests of the interactions in the special case of a $2 \times s$ table, giving a check on the constants.

If the class numbers are proportionate only slight modifications are necessary in the methods of analysis appropriate to the case of equal class numbers.

In the case of a $2 \times 2 \times 2 \times \dots$ table all estimates and all tests of significance may be made very simply whether interactions are assumed non-existent or not.

Tables with only slight inequalities in the class numbers may be analyzed by an approximate method based on the assumption that the variances of all the sub-class means are equal.

CONTINGENCY TABLES INVOLVING SMALL NUMBERS AND THE
 χ^2 TEST.

By F. YATES, B.A.

Introduction.

THERE has in the past been a good deal of argument as to the appropriate statistical tests of independence for contingency tables, particularly those in which each classification is a simple dichotomy (*i.e.* 2×2 tables). It is probably now almost universally admitted that when the numbers in the various cells are large the χ^2 test, introduced by K. Pearson in 1900,⁴ with the vital modification as to degrees of freedom established by R. A. Fisher in 1922,¹ and confirmed by Yule,⁵ is the appropriate one. The necessity for this modification has been amply demonstrated both from theoretical considerations^{1, 5} and by actual tests on material known to be almost if not quite free from association,^{2, 5} and there is no need to discuss the matter further here. Several other forms of test for 2×2 tables have been shown to be equivalent to the χ^2 test.¹

The χ^2 test is admittedly approximate, for in order to establish the test it is necessary to regard each cell value as normally distributed with a variance equal to the expected value, the whole set of values being subject to certain restrictions. The accuracy of this approximation depends on the numbers in the various cells, and in practice it has been customary to regard χ^2 as sufficiently accurate if no cell has an expectancy of less than 5. It is with the question of the applicability of χ^2 to 2×2 contingency tables involving small expectancies that we are directly concerned in this paper.

It was suggested to me by Professor Fisher that the probability of any observed set of values in a 2×2 contingency table with given marginal totals can be exactly determined. The method will be explained in the next section. Armed with the exact distribution the divergence of the χ^2 test in any special case can be tested. It will be shown that although the test as ordinarily applied becomes inaccurate even with moderately small numbers in the cells, a simple modification enables the range of usefulness to be considerably extended.

The problem of testing the independence of contingency tables involving small numbers is of considerable practical importance

in many branches of biological experimentation, where supplies of experimental material are limited. Medicine offers an extreme example, where considerations quite other than those of expense serve to limit the number of subjects available for testing a new treatment or method of operation. We have therefore thought it worth while to investigate the errors of the modified test in some detail. The results of this investigation are presented in the form of a table which serves to extend the range of this test still further.

In the case of contingency tables involving more than one degree of freedom, reasons are given for believing that the ordinary χ^2 test is considerably more reliable. As an example the exact distribution is worked out for a 2×3 table, and the agreement with the distribution given by χ^2 is shown to be good.

2×2 Contingency Tables.

We will define the table by the following notation :

	A	not A	Total
B	a	b	$N - n$
not B	c	d	n
Total	$N - n'$	n'	N

where $n \leq n' \leq \frac{1}{2}N$. It is easily seen that d can take all integral values from 0 to n . We now propose to find the frequency distribution of these values when the table is regarded as consisting of two samples of $N - n$ and n respectively from a binomial distribution with probability p of A occurring, subject to the restriction that the total of A in both samples shall be $N - n'$.

The probability of obtaining the values a and b in the sample of $N - n$ is

$$\frac{(N - n)!}{a! b!} p^a (1 - p)^b,$$

and of obtaining the values c and d in the sample of n is

$$\frac{n!}{c! d!} p^c (1 - p)^d.$$

The combined probability is therefore

$$\frac{(N - n)! n!}{a! b! c! d!} p^{a+c} (1 - p)^{b+d},$$

where a , b , c , and d are subject to the restrictions

$$a + b = N - n, \text{ and } c + d = n.$$

If the additional restriction that $a + c = N - n'$ or $b + d = n'$ is imposed, only those terms of the combined probability series must be selected which satisfy the restriction. But the factors

containing p are now constant, and therefore the required probabilities are equal to

$$\frac{1}{a!b!c!d!} / \sum_{d=0}^d \frac{1}{a!b!c!d!}$$

subject to the above restrictions.

The summation is easily performed, for it will be given by the coefficient of p^{a+c} in the expansion of

$$(p+q)^{N-n} (p+q)^n / (N-n)! n!,$$

i.e. of $p^{N-n'}$ in

$$(p+q)^N / (N-n)! n!.$$

This is

$$\frac{N!}{n!n'!(N-n)!(N-n')!},$$

The successive probabilities from $d=0$ to $d=n$ are therefore

$$\frac{(N-n)!(N-n')!}{N!(N-n-n')!}, \frac{(N-n)!(N-n')!n \cdot n'}{N!(N-n-n'+1)!1!},$$

$$\frac{(N-n)!(N-n')!n(n-1)n'(n'-1)}{N!(N-n-n'+2)!2!}, \dots, \frac{(N-n)!(N-n')!}{N!(n'-n)!}.$$

The successive probabilities are therefore proportional to the terms of the hypergeometric series $F(-n, -n', N-n-n'+1, 1)$.

Alternatively stated the probability corresponding to any term a, b, c, d , is

$$\frac{n!n'!(N-n)!(N-n')!}{N!a!b!c!d!},$$

i.e., the product of the factorials of the four marginal totals divided by the product of the factorials of the grand total and the four cell numbers.

In cases where N is not too large the distribution with any particular numerical values of the marginal totals can be computed quite quickly, using a table of factorials to determine some convenient term, and working out the rest of the distribution term by term, by simple multiplications and divisions. If a table of factorials is not available we may start with any convenient term as unity, and divide by the sum of the terms so obtained.

The numerical distribution could be used to provide a direct test of significance, but even when the marginal totals are quite small the evaluation of χ^2 is much more expeditious, and it is therefore of some interest to determine the limits within which χ^2 may be safely employed.

Validity of Assumption of Constancy of Marginal Totals.

No demonstration has yet been given that we are justified in assuming the constancy of the marginal totals. From one point

of view the matter is indeed almost obvious, for there is no inherent distinction between the rows and columns of the table, and if the marginal totals of the rows may be assumed fixed, then as an alternative the totals of the columns may equally be assumed fixed. If the two tests of significance arising from these separate assumptions are to be identical, then the test must ultimately involve the fixity of all marginal totals. But since it is perhaps not immediately clear that the tests of significance need be identical it may be worth while to examine the matter a little more closely.

All possible sets of observations having $N - n$ and n as the totals of the rows can be classified according to the value of n' . For each n' a 2.5 per cent. (or other) level of significance can be assigned for each tail (subject to discontinuity) and all sets of observations falling outside these limits will be counted significant. If 5 per cent. of each class is judged significant, then 5 per cent. of the whole population of sets will be judged significant, whatever the differences in the relative frequencies of the various classes. Clearly, therefore, the proposed test of significance is a valid test, in the sense that if there is no association a fixed proportion of the sets of observation will be judged significant, whatever p .

Leaving aside the question of discrimination between the two tails it is clear that when all the marginal totals are fixed the test is efficient, in the sense that if p and p' differ, the probability of obtaining a verdict of significance is as great as possible. For there is then only one degree of freedom, which must give rise to a definite distribution, of which the tails will provide the appropriate regions of significance.

That the marginal totals of the columns are necessarily assumed constant can be established without appeal to symmetry. Instead of regarding the table as generated by two random samples of $N - n$ and n respectively from a population with probability p , we may regard it as formed of a single sample N from such a population. In this sample each observation will be either A or not $-A$. The observations of class B may then be obtained by a random drawing of $N - n$ from this sample, for if there is no association there can be nothing in the attributes of any single observation which will determine whether it is also B or not $-B$. The remaining n observations will be consigned to class not $-B$. By this procedure of constructing the table we secure the fixity of all the marginal totals.

It is interesting to note that the marginal totals are in the nature of ancillary statistics as defined by Fisher. They determine the accuracy of the information supplied, but they are not subject to error or other variation themselves. From what has already been

said it will be apparent that there is no need to estimate p in order to make a test of significance. The distribution established in the previous section is independent of the actual value of p .

If a verdict of non-significance is obtained and this is regarded as conclusive, *i.e.* if we are prepared to assume that p and p' are identical, then the efficient (indeed sufficient) estimate of p is given by $(N - n')/N$. If we are not prepared to make this assumption, or if the probabilities are judged significantly different, the sufficient estimates of p and p' are $a/(N - n)$ and c/n , and the interpretation of the difference between them can be determined in the light of their numerical values and the nature of the data.

Binomial Distributions with known p .

The examination of the χ^2 test in the case of simple binomial distributions with known p will illustrate some important points.

We will first consider the symmetrical distribution .

$$(\frac{1}{2} + \frac{1}{2})^{10}.$$

Such a distribution would be obtained, for example, for the number of heads in groups of 10 tosses with a coin.

TABLE I.

Successes.	p' .	P .	$P(\chi)$.	Discrepancy.	$P(\chi')$.	Discrepancy.
0 10	0.0010	0.0010	0.0008	— 0.0002	0.0022	+ 0.0012
1 9	0.0098	0.0108	0.0057	— 0.0051	0.0134	+ 0.0027
2 8	0.0439	0.0547	0.0290	— 0.0257	0.0569	+ 0.0022
3 7	0.1172	0.1719	0.1030	— 0.0689	0.1714	— 0.0005
4 6	0.2051	0.3770	0.2635	— 0.1135	0.3759	— 0.0011
5	0.2461	—	—	—	—	—

Table I shows this distribution (which is symmetrical about 5 successes). p' is the probability of obtaining a given number of successes, and P the summation of p' from either tail, *i.e.* the probability of obtaining a number of successes deviating from the expected number 5 by a given or any greater amount in one direction. Thus the probability of obtaining exactly 8 successes is 0.0439, and the probability of obtaining 8 or more successes is 0.0547.

The probabilities given by the ordinary χ^2 test are given under the heading $P(\chi)$. These are one-half the ordinary χ^2 probabilities, so as to be comparable with a single tail of the distribution. In the computation of χ^2 the number of failures as well as the number of successes must be taken into account, giving a value for 8 successes, for example, of $3^2/5 + 3^2/5$ or 3.6. Since χ is distributed normally for one degree of freedom the exact probability correspond-

ing to this value of χ can be obtained by taking the square root and using a table of normal probability integral.

It will be seen that χ^2 always under-estimates the probability, the discrepancies being quite large except at the extreme tails. The true probability of 8 or more successes, for example, is 0.0547, whereas χ gives a value of 0.0290. We should thus on the χ^2 test judge such a deviation as almost reaching the 5 per cent. level of significance (2.5 per cent. for one tail), whereas in reality its probability of occurrence is over 10 per cent.

These discrepancies are primarily due to the fact that χ is a continuous distribution, whereas the distribution it is endeavouring to approximate is discontinuous. If we group the χ distribution, taking the half units of deviation from expectation as the group boundaries, we may expect to obtain a much closer approximation to the true distribution. This is equivalent to computing the values of χ^2 for deviations half a unit less than the true deviations, 8 successes, for example, being reckoned as $7\frac{1}{2}$, 2 as $2\frac{1}{2}$. This correction may be styled the *correction for continuity*, and the resultant value of χ denoted by χ' .

The resultant probabilities are shown under the heading $P(\chi')$. The agreement is now remarkably good, although the expectation of successes is only 5. χ' somewhat over-estimates the probability at the tails, and under-estimates it near the centre of the distribution. Only for the extreme values is the discrepancy really large, relative to the true probability, but inasmuch as in tests of significance we are generally concerned with the region (of one tail) of between 0.5 per cent. and 2.5 per cent., this is not of importance. In the critical region the discrepancies of this distribution are almost sufficiently small to be neglected in ordinary tests of significance, and were it only with distributions of this type that we had to deal we might leave the matter here.

There is, however, another source of discrepancy which does not appear in the above example. The χ distribution for one degree of freedom is necessarily symmetrical in terms of deviations from the expected values, whereas many of the distributions which we wish to test are not. Table II exhibits the binomial distribution

$$(\frac{3}{4} + \frac{1}{4})^{20}.$$

Here, as before, $P(\chi)$, in general, seriously under-estimates the probabilities. $P(\chi')$, however, is not now an adequate approximation in the critical region.

The magnitude of the errors to which $P(\chi')$ is subject in the various types of distribution will be discussed in the next section. Here it is sufficient to point out that $P(\chi')$ over-estimates the probability on the shorter tail, and under-estimates it on the longer tail, these

TABLE II.

Successes.	p' .	P .	$P(\chi)$.	Discrepancy.	$P(\chi')$.	Discrepancy.
0	0.0032	0.0032	0.0049	+0.0017	0.0102	+0.0070
1	0.0211	0.0243	0.0192	-0.0051	0.0351	+0.0108
2	0.0669	0.0912	0.0618	-0.0294	0.0985	+0.0073
3	0.1339	0.2251	0.1515	-0.0736	0.2192	-0.0059
4	0.1897	0.4148	0.3129	-0.1019	0.3983	-0.0165
5	0.2023	—	—	—	—	—
6	0.1686	0.3829	0.3129	-0.0700	0.3983	+0.0154
7	0.1124	0.2143	0.1515	-0.0628	0.2192	+0.0049
8	0.0609	0.1019	0.0618	-0.0401	0.0985	-0.0034
9	0.0271	0.0410	0.0192	-0.0218	0.0351	-0.0059
10	0.0099	0.0139	0.0049	-0.0090	0.0102	-0.0037
11	0.0030	0.0040	0.0010	-0.0030	0.0023	-0.0017
12	0.0008	0.0010	0.0001	-0.0009	0.0004	-0.0006
13	0.0002	0.0002	—	—	—	—

discrepancies being reversed near the centre of the distribution. In symmetrical and nearly symmetrical distributions $P(\chi')$ over-estimates the probabilities at both tails and under-estimates them near the centre of the distribution. Such discrepancies, however, are small compared with those arising in violently unsymmetrical cases.

From the nature of these discrepancies it is clear that no simple modification of the correction for continuity will materially improve the approximations obtained.

Discrepancies of the χ^2 Test after correcting for Continuity.

If any given contingency distribution is calculated by means of the appropriate hypergeometric series the discrepancies between the values of χ corresponding to the true probabilities and the equivalent values of χ' can be evaluated. These discrepancies are more convenient to work with than the discrepancies between the true and χ' probabilities which were considered in the last section: clearly they are immediately convertible the one into the other by reference to a table of the normal probability integral.

There are, of course, in general no discrepancies corresponding to the exact 2.5 per cent. and 0.5 per cent. points, but it is possible to determine approximate hypothetical discrepancies corresponding to these points on the true probability scale by interpolation. If the discrepancies are plotted against the logarithms of the true probabilities the resultant points lie on remarkably regular curves, making graphical or other interpolation easy. Fig. 1 shows the graph of these discrepancies in the case of the binomial of Table II.

For every distribution generated by a 2×2 contingency table with fixed marginal totals but variable class numbers, therefore, a

hypothetical discrepancy in χ' for any given level of significance can be obtained. Clearly the variety of contingency tables met with in practice is very large, but the variation of the χ' discrepancy can be exhibited in quite a compact table in sufficient detail for practical purposes.

The distribution generated by any contingency table with fixed

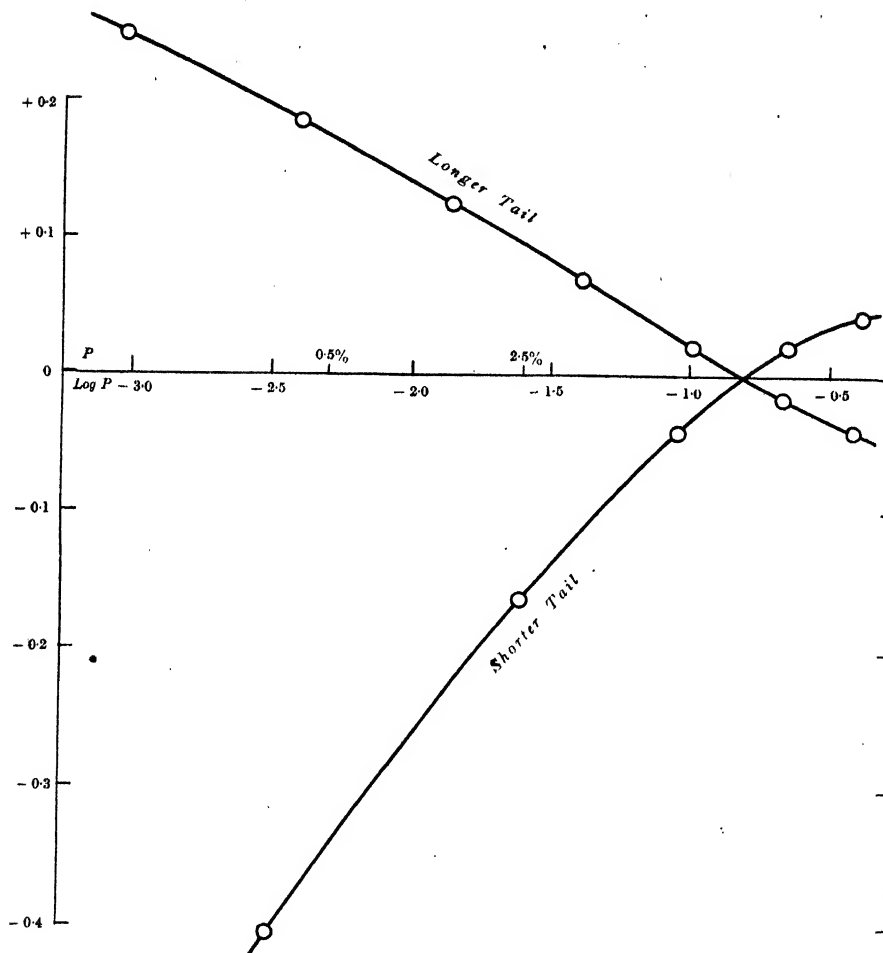


FIG. 1.—Discrepancies of χ' for binomial $(\frac{1}{4} + \frac{3}{4})^{20}$.

marginal totals may be defined in various ways. We might, for instance, use the marginal totals (only three of which are independent) for this purpose, but any three functions of them will do equally well.

There is, in fact, a three-dimensional field of distributions and any

position in this field can be defined by three co-ordinates. For our purpose it will be most convenient to use co-ordinates which represent properties of the distribution rather than of the table.

Consider the general table discussed in the second section. The distribution will have $n + 1$ terms corresponding to the $n + 1$ values from 0 to n of d . The expectation for d will be nn'/N , and this will always be less than $\frac{1}{2}n$. All distributions, therefore, may be classified according to the smallest expectation of the table, and according to the number of terms or range. In each class there will be a whole series of distributions having fixed expectation m and range $n + 1$, but with varying N (and n'). N and n' can, in fact, assume all integral solutions of

$$m = nn'/N.$$

with $n' \geq n$. The contingency distribution with smallest possible N will be called the *limiting contingency distribution* of that class. The smallest N must be equal to or greater than n^2/m , but there is no upper limit. As N tends to infinity the distribution approximates to the binomial distribution

$$(p + q)^n,$$

where $p = m/n$, i.e. the expectation divided by the range less one. In what follows it will be convenient to use m and p instead of m and $n + 1$ as defining the class.

With expectation 4 and range $12 + 1$, for example, we obtain the series with sets of marginal totals (24, 12; 24, 12), (27, 12; 26, 13), (30, 12; 28, 14), etc., with the limiting binomial $(\frac{1}{3} + \frac{2}{3})^{12}$. With expectation $3\frac{1}{2}$ and range $10 + 1$ we obtain the series (30, 10; 26, 14), (50, 10; 39, 21), etc. In each case the first-named distribution is the limiting contingency distribution.

The utility of the above classification lies in the fact (established by examination of special cases) that the χ' discrepancies are similar for all distributions in any one class, and in general decrease or increase steadily with increasing N . Thus the knowledge of the χ' discrepancy for the limiting contingency distribution and the binomial of any class sets definite limits to its value for any distribution of that class.

Moreover the χ' discrepancies for the limiting contingency distributions and the binomials vary in a regular manner as m and p are varied. Fig. 2 illustrates this variation with variations of p when m is equal to 4. There are four separate diagrams corresponding to 2.5 per cent. and 0.5 per cent. points of the longer and shorter tails. In each case the values actually calculated are marked. It will be seen that the values fall very satisfactorily on to smooth curves (the curves of binomial values are shown full, those of the limiting contingency series values dotted).

Practically we are only interested in the values of p between 0 and 0.5, but higher values of p have been included where possible to illustrate the continuity. In the series of limiting contingency

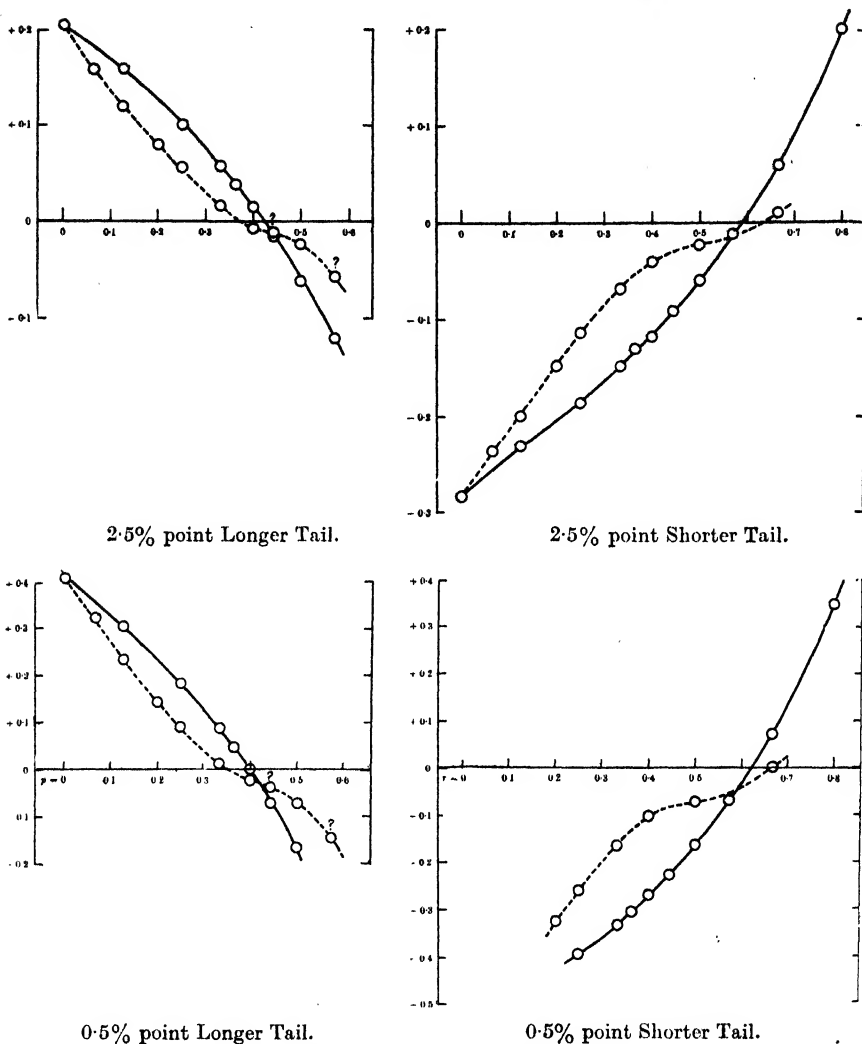


FIG. 2.—Discrepancies of χ^2 , expectation 4.

The vertical scales represent discrepancies, the horizontal scales values of p . The full curves are those given by the binomials, the dotted curves those given by the limiting contingency distributions.

distributions only values such that $N = n^2/m$ have been included, except in the case of the points marked with a query, where no

integral values of N exactly equal to n^2/m were available, but the points given by the next higher integral values appeared of sufficient interest for conclusion.

These diagrams are similar to those obtained with other expectations, subject to the proviso that the larger the expectation the smaller the discrepancies. Several interesting facts may be at once deduced from them. Thus of all the binomial distributions with given expectation and $p \leq 0.5$ the Poisson distribution ($p = 0$) gives the greatest discrepancies, the discrepancies in χ' for expectation 4 being $+0.205$ and -0.283 for the 2.5 per cent. point on the longer and shorter tail respectively, corresponding to χ' probabilities of 1.52 per cent. and 4.68 per cent. respectively. Also the discrepancies on the two tails of distributions with small p are approximately equal but of opposite sign. The discrepancies of the symmetrical binomial ($p = 0.5$), on the other hand, are quite small, namely -0.060 with a corresponding probability of 2.87 per cent. Thus asymmetry is the most powerful disturbing factor of the χ' test. This confirms the conclusion of the fourth section.

The discrepancies of the limiting contingency series are less than those of the corresponding binomial series except for a small region in the neighbourhood of zero discrepancy. In general, therefore, the binomial may be regarded as furnishing the upper limits, and the limiting contingency distribution the lower limits to the discrepancies with any given m and p .

The application of the above results to the construction of practical tests of significance will now be considered. Clearly if it is desired to test the significance of a table with some particular marginal totals (that is, with some particular m , p and N) and the 2.5 per cent. and 0.5 per cent. values of χ' for the corresponding distribution are known, the test can be made immediately by comparing the value of χ' calculated from the table with the 2.5 per cent. and 0.5 per cent. values. Ideally, therefore, a table of the 2.5 per cent. and 0.5 per cent. values of χ' for each tail is required, covering all possible contingency distributions. This would be a three-dimensional table, and we have not attempted to construct it. Instead, two two-dimensional tables have been computed, one giving the values of χ' for the binomial distributions, the other those for the limiting contingency distributions. Both the 2.5 per cent. and the 0.5 per cent. points are tabulated for both tails for values of m ranging from 1 to 96 and values of p equal to 0, 0.25, and 0.5. Since the longer and shorter tail are identical when $p = 0.5$ and the binomial and limiting contingency distribution are identical when $p = 0$, the whole set of values for either the 2.5 per cent. or 0.5 per cent. points can be most conveniently given in a single table. This is done

in Table III. In the case of the binomial with $m = 5$, $p = 0.25$, for instance, Fig. 1 shows that the discrepancies for the 2.5 per cent. and 0.5 per cent. points are -0.162 and -0.343 on the shorter

TABLE III.

The 2.5 per cent. and 0.5 per cent. points of χ' .

The values for the binomial distributions are shown in ordinary type, those for the limiting contingency distributions in italics.

m = the smallest expectation. p = $\frac{\text{the smallest expectation}}{\text{the smallest marginal total}}$

$m \backslash p$	2.5 per cent. points.					0.5 per cent. points.				
	Longer tail.			Shorter tail.		Longer tail.			Shorter tail.	
	0.	0.25.	0.5.	0.25.	0.	0.	0.25.	0.5.	0.25.	0.
1	2.32	2.10 <i>2.04</i>	— —	— —	—	3.33	2.79 <i>2.67</i>	— —	— —	—
2	2.24	2.08 <i>2.03</i>	— <i>1.91</i>	— —	—	3.13	2.79 <i>2.67</i>	— —	— —	—
3	2.19	2.07 <i>2.02</i>	1.88 <i>1.93</i>	1.73 <i>1.83</i>	—	3.05	2.78 <i>2.67</i>	— <i>2.48</i>	— —	—
4	2.16	2.06 <i>2.02</i>	1.90 <i>1.94</i>	1.77 <i>1.85</i>	1.68	2.97	2.76 <i>2.67</i>	2.41 <i>2.50</i>	2.18 <i>2.32</i>	—
5	2.14	2.06 <i>2.01</i>	1.91 <i>1.94</i>	1.80 <i>1.86</i>	1.71	2.95	2.75 <i>2.66</i>	2.44 <i>2.52</i>	2.23 <i>2.36</i>	2.06
6	2.13	2.05 <i>2.01</i>	1.92 <i>1.94</i>	1.82 <i>1.87</i>	1.74	2.92	2.73 <i>2.66</i>	2.47 <i>2.53</i>	2.27 <i>2.38</i>	2.13
8	2.11	2.04 <i>2.00</i>	1.93 <i>1.95</i>	1.84 <i>1.89</i>	1.77	2.88	2.72 <i>2.65</i>	2.50 <i>2.54</i>	2.32 <i>2.42</i>	2.19
12	2.08	2.02 <i>1.99</i>	1.94 <i>1.95</i>	1.87 <i>1.90</i>	1.81	2.83	2.70 <i>2.64</i>	2.52 <i>2.55</i>	2.38 <i>2.46</i>	2.27
24	2.05	2.01 <i>1.99</i>	1.95 <i>1.95</i>	1.90 <i>1.92</i>	1.86	2.76	2.67 <i>2.63</i>	2.55 <i>2.56</i>	2.45 <i>2.50</i>	2.37
48	2.03	2.00 <i>1.98</i>	1.96 <i>1.96</i>	1.91 <i>1.94</i>	1.89	2.70	2.64 <i>2.62</i>	2.56 <i>2.57</i>	2.49 <i>2.52</i>	2.43
96	2.01	1.99 <i>1.97</i>	1.96 <i>1.96</i>	1.93 <i>1.94</i>	1.91	2.67	2.63 <i>2.60</i>	2.57 <i>2.57</i>	2.52 <i>2.54</i>	2.48

tail, and $+0.095$ and $+0.173$ on the longer tail. Adding the 2.5 per cent. discrepancies to 1.960 (the 2.5 per cent. point of χ) and the 0.5 per cent. discrepancies to 2.576 (the 0.5 per cent. point of χ) gives the tabulated values of 1.80, 2.23, 2.06 and 2.75 respectively.

Since the table only contains values for the binomials and the limiting contingency distributions it only serves to provide upper and lower limits to the actual 2.5 per cent. and 0.5 per cent points of χ' for other contingency distributions. In testing any particular

contingency table interpolation will be necessary to determine these upper and lower limits, unless the m and p of the table to be tested happen to coincide with the tabulated values. There is, however, no need for any great accuracy in this interpolation, which can be made more or less by inspection.

Should the actual value of χ' calculated from the particular contingency table being tested happen to fall between the upper and lower limits of either the 2.5 per cent. or 0.5 per cent. points, then the appropriate hypergeometric series must be calculated, at least if an exact test is required.

Table III also serves to determine the limits of applicability of the χ' test when the standard χ^2 distribution for one degree of freedom is used (*i.e.* of the ordinary χ^2 test after correction for continuity). Thus if it is considered that errors in the χ' test up to ± 0.5 per cent. may be tolerated when the true probability is 2.5 per cent., the limits of the 2.5 per cent. point of χ' given by Table III must be 2.054 and 1.881, these being the 2.0 per cent. and 3.0 per cent. points of the ordinary χ distribution. When p equals 0 (the worst case), these limits are exceeded when m is less than 40. In the symmetrical distributions ($p = 0.5$) m can be as small as 3. At these limits the relative discrepancies at the 0.5 per cent. points are decidedly greater, for the χ' probabilities range between 0.8 per cent. and 0.3 per cent. roughly.

Table IV gives the limits for permissible discrepancies of ± 0.5 per cent. and ± 0.25 per cent. at the 2.5 per cent. points.

TABLE IV.

Limits of m within which the standard χ^2 table may be used.

Permissible discrepancy at 2.5% point.	$p = 0.5.$	$p \geq 0.375.$	$p \geq 0.25.$	$p \geq 0.$
$\pm 0.5\%$	$m \geq 3$	$m \geq 7$	$m \geq 16$	$m \geq 40$
$\pm 0.25\%$	$m \geq 6$	$m \geq 20$	$m \geq 75$	$m \geq 125$

In view of the wide variation in the limits of m for different p it will probably be more convenient in practice to use Table III if it is available whenever the smallest expectation is less than 100. On the other hand, when Table III is not available the worker will not be led badly astray if he applies the ordinary χ^2 test (after correcting for continuity) to tables giving expectations as low as 10, so long as the corresponding distributions are reasonably symmetrical. Moreover, in view of the fact that the discrepancies are of opposite signs on the two tails of the distribution, no consistent under- or over-estimates of significance will be made when applying

the ordinary test corrected for continuity to a heterogeneous collection of data, even with very small expectations.

The correction for continuity involves practically no extra work and should therefore be applied unless the expectations are very large. Even with a smallest expectation of 500 the discrepancy introduced into the χ^2 test at the 2.5 per cent. point of either tail by omitting this correction may be as much as 0.26 per cent.

The sequence of operations in making a practical test of any 2×2 table is therefore as follows :

1. If the smallest expectation is less than 500, calculate χ'^2 , i.e. χ^2 corrected for continuity, instead of χ^2 .

2. If χ'^2 is in the neighbourhood of the 5 per cent. or 1 per cent. point and the smallest expectation is less than 100, calculate p , take the square root of χ'^2 , and refer to Table III, interpolating if necessary. (Note that interpolation will often be unnecessary because the value of χ' calculated from the observations is greater or less than all the values of Table III between which interpolation would be made.) Alternatively Table IV may first be consulted to decide if reference to Table III is really necessary.

3. If the value of χ' calculated from the observations lies between the upper and lower limits given by Table III and a more precise test is desired, calculate the hypergeometric series and perform the exact test.

Example. The following figures for malocclusion of the teeth in infants were obtained by M. Hellman,³ who draws from them the conclusion that bottle-feeding is one of the factors causing malocclusion.

	Normal Teeth.	Malocclusion.	Total.
Breast-fed	4	16	20
Bottle-fed	1	21	22
Breast and bottle fed	3	47	50
Total	8	84	92

Considering first the wholly breast-fed and wholly bottle-fed only we have the fourfold table :

	Normal Teeth.	Malocclusion.	Total.
Breast-fed	4	16	20
Bottle-fed	1	21	22
Total	5	37	42

Here
$$\chi^2 = \frac{(4 \times 21 - 1 \times 16)^2 \cdot 42}{5 \cdot 37 \cdot 20 \cdot 22} = 2.386,$$

$$\chi = 1.545, P(\chi) = 0.0612, P(\chi^2) = 2P(\chi) = 0.1224$$

$$\chi'^2 = \frac{(3\frac{1}{2} \times 20\frac{1}{2} - 1\frac{1}{2} \times 16\frac{1}{2})^2 \cdot 42}{5 \cdot 37 \cdot 20 \cdot 22} = 1.140,$$

$$\chi' = 1.068, P(\chi') = 0.1427, P(\chi'^2) = 2P(\chi') = 0.2854.$$

Note that $P(\chi)$ and $P(\chi')$ are obtained from χ and χ' by reference to a table of the normal probability integral.

The exact distribution is computed as follows. The probability of obtaining no normal breast-fed children is

$$\frac{5! \cdot 37! \cdot 20! \cdot 22!}{42! \cdot 0! \cdot 20! \cdot 5! \cdot 17!} = 0.0309568.$$

Using the successive multipliers shown we obtain the complete table:

No. of Normal Breast-fed Children.	Multiplier.	Probability.
0	—	0.0309568
1	5.20/1.18	0.171982
2	4.19/2.19	0.343964
3	3.18/3.20	0.309568
4	2.17/4.21	0.125301
5	1.16/5.22	0.018226
		0.143527
		0.999998

Thus the true probability of obtaining four or more normal breast-fed children is 0.1435. $P(\chi')$ gives 0.1427, an excellent approximation, whereas $P(\chi)$ gives 0.0612, which, though not in itself attaining significance, is less than half the true value; this would be exceedingly misleading if a number of such probabilities from different classes of experiment were to be combined.

χ' is so far from the 2.5 per cent. point for any m and p that exact reference to Table III is unnecessary. The smallest value of the 2.5 per cent. of χ' in Table III is 1.68.

If malocclusion is due to mechanical action on the teeth it may be considered that children both breast-fed and bottle-fed are exposed to much the same risk of damage as those wholly bottle-fed. Combining these two classes we have the following table:

	Normal Teeth.	Malocclusion.	Total.
Breast-fed	4	16	20
Bottle or breast and bottle fed ...	4	68	72
Total	8	84	92

This table gives the following results :

$$\chi^2 = 4.113, \chi = 2.028, P(\chi) = 0.0213.$$

$$\chi'^2 = 2.495, \chi' = 1.580, P(\chi') = 0.0571.$$

No. of Normal Breast-fed Children.	Probability.
0	0.12859
1	0.31652
2	0.31892
3	0.17136
4	0.05355
5	0.00993
6	0.00106
7	0.00006
8	0.00000
	0.99999

Here the ordinary χ^2 test attains the 5 per cent. level of significance (2.5 per cent. on one tail). The true probability, however, 0.0646 on the one tail, is nowhere near this, and again the correction for continuity, which gives a probability of 0.0571, is a good approximation. Here also χ' is sufficiently small to make reference to Table III unnecessary.

Thus it will be seen that even on the most favourable grouping of the data association of the degree observed might have arisen by chance about once in eight times, so that Hillman's conclusions cannot be regarded as established.

In neither of these examples has it been necessary to make any exact reference to Table III. This is the case with the great majority of tests, but to illustrate the application of the table we will suppose that the results obtained in the second case were as follows :

	Normal Teeth.	Malocclusion.	Total.
Breast-fed*	5	15	20
Breast or breast and bottle fed ...	3	69	72
Total	8	84	92

Here $\chi' = 2.477$ and $P(\chi') = 0.0066$, which on the face of it indicates significance approaching the 0.5 per cent. point, but since the smallest expectation $m = 8 \times 20/92 = 1.74$, which is very small, and $p = 1.74/8 = 0.22$, so that the distribution will be decidedly skew, some assurance is needed that the approximation is good enough. Referring to Table III we see that for $m = 1$ and $p = 0$ the 2.5 per cent. point of χ' on the longer tail is 2.32, for $p = 0.25$ it lies between

2.10 and 2.04, and for $m = 2$ the corresponding values are 2.24, 2.08, and 2.03. The value 2.477 calculated from the observations is greater than all these, and therefore the 2.5 per cent. level of significance is attained. The 0.5 per cent. level of significance, on the other hand, is not attained, since the value 2.477 is less than all the values of the 0.5 per cent. point between which interpolation would have to be made. The true value of the probability given by the exact distribution already worked out is 0.0110.

Contingency Tables involving more than one Degree of Freedom.

The exact method of determining the probabilities of every set of values conforming to given marginal totals can easily be extended to tables with more than two classes in either or both classifications. The probability of obtaining any given set of values is, as in the case of 2×2 tables, the product of the factorials of all the marginal totals divided by the product of the factorials of the grand total and the individual cell numbers.

In the case of a 3×2 table, for instance, which involves two degrees of freedom, the probabilities of each particular set of values can be set out in a two-dimensional diagram which consists of two sets of interlocking hypergeometric series. The computation naturally becomes tedious if the number of possible sets of values is at all large, but it involves no new principle.

The discontinuous nature of the distribution has in general not so serious an effect on the χ^2 test. For even with quite small marginal totals there are far more possible sets of values, and it is only in exceptional cases that more than one set of values has the same χ^2 . If we regard the probability of obtaining any one set of values as the result of grouping a continuous frequency distribution, the χ^2 distribution may be considered as an approximation to this continuous distribution. With any considerable deviation from expectation the surfaces of equal χ^2 cut across many of the cells representing the sets of values; some of these cells will be included in the true probability for that χ^2 , and others excluded. Inasmuch, however, as the probability density falls off with increasing χ^2 , χ^2 may as before be expected to under-estimate the true probability except at the tails, where other sources of disturbance become important.

As an illustration of what is likely to occur in practice take the contingency table—

a_1 b_1	a_2 b_2	a_3 b_3	17 13
13	11	6	30

TABLE V.
Discrepancies in a 2×3 Contingency Table.

a_1 .	a_2 .	χ^2 .	P .	$P(\chi^2)$.
9	2	4.750	0.1067	0.0930
7	1	4.963	0.0948	0.0836
8	1	5.192	0.0830	0.0746
5	6	5.736	0.0724	0.0568
6	1	6.102	0.0658	0.0473
3	4	6.285	0.0592	0.0432
6	6	6.363	0.0532	0.0415
3	5	6.442	0.0483	0.0399
8	5			
4	6	6.476	0.0365	0.0392
9	4	6.630	0.0317	0.0363
9	1	6.786	0.0268	0.0336
4	2	7.311	0.0221	0.0258
3	3	8.113	0.0188	0.0173
10	2	8.338	0.0167	0.0155
7	6	8.358	0.0149	0.0153
3	6	8.583	0.0130	0.0137
5	1	8.608	0.0120	0.0135
10	3	8.914	0.0094	0.0116
10	1	9.748	0.0081	0.0076
8	0	9.838	0.0071	0.0073
7	0	10.236	0.0061	0.0060
2	5	10.546	0.0053	0.0051
9	5			

Table V gives the true probabilities in the critical region of obtaining as great or greater χ^2 than that given in the first column. $P(\chi^2)$ is given in the third column. It will be noted that χ^2 here serves as a criterion by which deviations from expectation may be arranged in order of magnitude, and performs an additional and entirely different function to that in the case of a single degree of freedom where its *only* function has been to serve as an approximation to the actual distribution. It could, however, have been made to perform this additional function in the case of a single degree of freedom, when it would serve to combine the deviations of the two tails on the basis of equal χ^2 . This would eliminate the effects of skewness in the true distribution and the agreement would be much improved. The same elimination is undoubtedly effected in the case of more than one degree of freedom. Whether χ^2 is really the best criterion of a deviation from expectation we do not propose to discuss here.

The distribution given here is likely to be an example which is fairly favourable to χ^2 . Cases where some of the marginal totals are large, and others small, so that certain degrees of freedom approximate to the binomial or Poisson distributions, may be expected, in the light of the results obtained for one degree of freedom,

to give much more unfavourable results. Another source of discrepancy between the χ^2 and the exact probability is that due to several cells having the same value of χ^2 . This will occur in symmetrical tables, particularly those with integral expectations in the various cells.

Summary.

1. A method of obtaining the exact probability distribution associated with a 2×2 contingency table with given marginal totals is developed.

2. It is shown that the ordinary χ^2 test is liable to considerable errors when the expectations are moderately small. A simple modification is suggested which considerably increases the accuracy of χ^2 . Tables are given which enable the limits of applicability of the modified test to be determined, and serve as a means of increasing the accuracy of the modified test.

3. The applicability of the χ^2 test to contingency tables involving more than one degree of freedom is briefly discussed.

References.

¹ R. A. Fisher (1922). "On the Interpretation of χ^2 from Contingency Tables, and the Calculation of P ." *Journ. Roy. Stat. Soc.*, Vol. LXXXV, pp. 87-94.

² R. A. Fisher (1926). "Bayes' Theorem and the Fourfold Table." *Eugenics Rev.*, 18, pp. 32-33.

³ M. Hellman (1914). "A Study of some Etiological Factors of Malocclusion." *Dental Cosmos*, Vol. LVI, pp. 1017-1032.

⁴ K. Pearson (1900). "On the criterion that a given system of deviations from the probable in the case of a correlated system of variables is such that it can be reasonably supposed to have arisen from random sampling." *Phil. Mag.*, 1, Series 5, Vol. L, pp. 157-175.

⁵ G. U. Yule (1922). "On the Application of the χ^2 Method of Association and Contingency Tables, with Experimental Illustrations." *Journ. Roy. Stat. Soc.*, Vol. LXXXV, pp. 95-104.

THE VALUE OF A UNIFORMITY TRIAL IN FIELD EXPERIMENTATION WITH RUBBER.

By R. K. S. MURRAY, A.R.C.Sc.

(*Rubber Research Scheme, Ceylon;*
Research Laboratories, Culloden, Neboda, Ceylon.)

FIELD experiments are subject to two main potential sources of error: (1) variation due to outside influences such as differences in soil fertility, exposure to wind, disease, etc., and (2) the inherent variability of the material used. By the methods of plot arrangement evolved by Fisher these sources of error can be estimated with precision and given due weight in the analysis of the results. In an experiment where the heterogeneity of the soil or plant material is considerable, however, the residual error is high and the accuracy of the experiment correspondingly low. On the assumption that the disparity between the yields of the different plots is relatively constant, one would suppose that the value of such an experiment might be legitimately enhanced by utilising previous crop records. In the past the "percentage increase" method has sometimes been used for correcting the experimental yields, but this method is statistically unsatisfactory and may actually lead to a loss rather than a gain in precision.

The inference that the yield of a plot in one year is any guide to its yield in another implies that the expectation of yield is well represented in terms of the previous performance by a linear regression function. If the yields in the preliminary and experimental periods be denoted by x and y respectively, then the appropriate regression coefficient b is given by the ratio $\frac{\text{Cov}_{xy}}{V_x}$, where Cov_{xy} is the covariance of the preliminary and experimental yields, or the mean product of their deviations from their means, and V_x is the residual variance of the preliminary yields. The yields in the experimental period are to be adjusted by means of this coefficient, so that comparisons of adjusted yields become, in fact, comparisons of the quantities $(y - bx)$.

Now
$$(y - bx)^2 = b^2 x^2 - 2bxy + y^2,$$

so that, in obtaining the analysis of variance of the adjusted yields, the sum of squares in any line can be calculated by multiplying x^2 by b^2 , xy by $-2b$, and y^2 by unity, and adding the products.

178 *Uniformity Trials in Field Experiments with Rubber*

Sanders(1) showed that the error variance of the adjusted yields ($V_{y..x}$) can be conveniently calculated from the formula

$$V_{y..x} = V_y - \frac{(\text{Cov}_{xy})^2}{V_x},$$

the resultant figure being corrected for the one degree of freedom used in employing the regression. The increased precision gained by using the previous records as a uniformity trial is then given by the ratio $V_y : V_{y..x}$.

Sanders used this method for investigating a uniformity trial on annual crops in which each year's data referred to a different crop. In one field the relative plot yields showed no constancy, and the previous figures were therefore of no value. In another field, however, the differences between the plot yields did show a tendency to persist, and by using a regression on the mean yield of three previous years the precision of the experiment was increased by nearly 150 per cent. In view of the loss in time and increase in labour and expenditure incurred in compiling records in a previous year or years, it is doubtful, however, on the basis of these results, whether the method would usually justify itself for annual crops.

With a perennial crop from which the yields in successive years are derived from the same individual plants, a higher correlation is to be expected. Eden(2), working with tea, has demonstrated a striking reduction in error by utilising the results of preliminary pluckings. The precision of the experiment was increased more than sixfold, that is to say that without the previous records the replications would need to have been more than six times as numerous to achieve the same degree of accuracy.

Field experimentation with mature rubber (*Hevea brasiliensis*) has at present to be conducted on a seedling population of great variability; the annual yield of individual plants in any one field may, in extreme cases, vary from less than 1 lb. up to 100 lb. of rubber. The trees are planted at a density of approximately 100 per acre, and in order to keep soil heterogeneity within reasonable bounds it is desirable that the number of individual plants in a plot be relatively small. Lord(3) computed that the optimum plot size was approximately twenty-four trees. With such small plots the crop from an exceptional individual tree assumes important proportions, and the yields of the several plots are liable to show considerable diversity. The yield of an individual tree remains fairly constant from year to year, and a substantial benefit as the result of a previous uniformity trial may therefore be anticipated.

THE DATA.

While enjoying the hospitality of Rothamsted Experimental Station the writer had the opportunity to examine ten years' yield records of about 1000 individual rubber trees planted in Sumatra. These figures were given to the Statistical Department by Mr J. Grantham of the United States Rubber Plantations Incorporated, to whom the writer is indebted for permission to publish the results of the work. At the suggestion of Prof. R. A. Fisher, then Chief Statistician at Rothamsted, these figures were analysed with a view to determining the utility of previous crop records in field experimentation with rubber.

As stated above, the data consist of individual tree records of nearly 1000 ordinary seedling rubber trees, planted in 1911 and thinned out in 1918 and subsequently. From the chart accompanying the records a 5×5 Latin square of twenty-five tree plots was marked out, barrier rows being allotted as if a real test of five manurial treatments (replicated five-fold) were involved. The plot yields for any year were calculated from the yields of the individual trees in the several plots.

In order that the trial should be representative of results likely to be obtained with fully mature rubber, the last four years' records, *i.e.* 1926-9 were selected, the age of the trees being then 15-18 years. The method of enquiry was to regard 1926 as the preliminary year, and to determine how far the residual variance was reduced by utilising these yields with 1927, 1928 and 1929 respectively as supposedly experimental years.

Table I. *Yields for "Preliminary" year 1926.*

851	825	837	975	1015	4503
840	945	776	1108	970	4639
925	920	968	1009	1026	4848
869	1028	735	730	892	4254
979	931	1012	1070	989	4981
4464	4649	4328	4892	4892	23225

General mean = 929.0

Table II. *Yields for "Experimental" Year 1927.*

906	858	836	923	1023	4546
957	997	846	1126	995	4921
978	1042	1003	1061	1049	5133
956	1054	764	837	946	4557
1110	956	1095	1180	995	5336
4907	4907	4544	5127	5008	24493

General mean = 979.7

180 *Uniformity Trials in Field Experiments with Rubber*

The method is worked out in detail for the "experimental" year 1927. Tables I and II give the plot yields for the years 1926 and 1927 respectively, the yield figures given by Grantham being divided by 100 and expressed to the nearest whole number.

The analyses of variance for the two years are given in Tables III and IV.

Table III. *Analysis of variance "Preliminary" year 1926.*

Variance due to	Degrees of freedom	Sum of squares	Variance	S.D.
Rows	4	65,437	16359	
Columns	4	51,057	12764	
Error	16	125,998	7874.9	88.74
Total	24	242,492	10104	

Table IV. *Analysis of variance "Experimental" year 1927.*

Variance due to	Degrees of freedom	Sum of squares	Variance	S.D.
Rows	4	90,698	22674.5	
Columns	4	31,145	7786	
Error	16	118,744	7421.5	86.15
Total	24	240,587	10024	

Since there were no actual treatments, 16 degrees of freedom are used for the estimation of error. Despite the large variance eliminated by rows, the residual variance in 1927 is 7421.5, giving a standard error of a single plot of about 8.79 per cent., and for the total of five plots of about 3.93 per cent.

The analysis of covariance between 1927 and 1926 is obtained by using the sums of products for the two years instead of the sums of squares for either one year, and is as follows:

Table V. *Analysis of covariance 1927 × 1926.*

Variance due to	Degrees of freedom	Sum of products	Covariance
Rows	4	75,610	
Columns	4	38,872	
Error	16	105,874	6617.1
Total	24	220,356	

Substituting the figures for 1927 (y) and 1926 (x) in the formula given above, we obtain

$$V_{y.x} = V_y - \frac{(\text{Cov}_{xy})^2}{V_x} = 7421.5 - \frac{(6617.1)^2}{7874.9} = 1861.4.$$

But in employing a linear regression one degree of freedom is used up, and we have finally

$$V_{v.x} = \frac{1861.4 \times 16}{15} = 1985.5.$$

The residual variance has thus been reduced from 7421.5 to 1985.5, and the increased precision of the experiment is measured by the fraction

$$\frac{V_y}{V_{v.x}} = \frac{7421.5}{1985.5} = 3.74.$$

In other words, if the year 1927 were to stand alone, the number of replications necessary to achieve the same degree of accuracy would be 5×3.74 , or approximately 19.

The same procedure has been followed with the years 1928 and 1929, the regression in all cases being on the preliminary year 1926.

The following table gives the increased precision $V_y:V_{v.x}$ for the several years:

	$V_y:V_{v.x}$
1927 \times 1926	3.74
1928 \times 1926	2.52
1929 \times 1926	2.15

These figures show that a considerable gain in precision is effected by the application of this method, the biggest advantage being obtained when the experimental and preliminary years are consecutive. It is probable that a somewhat greater reduction in error would be achieved by employing a regression on the mean of a number of previous years, but in experimenting with rubber such figures are most unlikely to be available and it would probably be impracticable to delay the experiment for several years in order to obtain them.

The variance of the 1927 yields adjusted for 1926 is analysed in Table VI, using the equation

$$S(y - bx)^2 = S(y^2) - 2bS(xy) + b^2S(x^2).$$

Here b is the regression coefficient on the residuals after allowing for row and column effects, and is equal to

$$\frac{6617.1}{7874.9} = 0.840.$$

Table VI. *Analysis of variance of adjusted yield 1927/1926.*

Variance due to	Degrees of freedom	Sum of squares	Variance	S.D.
Rows	4	9,845	2461.2	
Columns	4	1,866	466.5	
Error	15	29,782	1985.5	44.56
Total	23	41,493	1804.0	

182 *Uniformity Trials in Field Experiments with Rubber*

The standard error of a single plot is now reduced from 8.79 per cent. to about 4.55 per cent., and of the total of five plots from 3.93 per cent. to about 2.03 per cent.

As an example of method the row totals for 1927 have been adjusted by subtracting from any experimental yield y the factor $b(x - \bar{x})$, where \bar{x} is the mean row total for 1926. The adjusted and actual figures are given in Table VII.

Table VII. *Row totals 1927.*

Actual totals	Adjusted totals
4546	4665
4921	4926
5133	4962
4557	4885
5336	5054

It is clear, at a glance, that the variation in the adjusted row totals is considerably less than in the actual totals, and a study of the analyses of variance of the original and corrected yields (Tables IV and VI) shows that whereas the original differences were significant by the z test, the adjusted differences are not so. This implies that whatever fertility or other differences were eliminated by rows are more or less constant over the two years, and are therefore equally well eliminated by the regression. This is the reverse of the results obtained by Eden⁽²⁾ with tea. Discussing the tea figures, Fisher⁽⁴⁾ points out that the elimination of positional variance by means of rows and columns is actually more important in the adjusted than in the unadjusted yields. With the figures presented in this paper, on the other hand, the error has actually been increased (though only by chance) by the removal of row and column effect.

A practical point to be noted is that the regression is a more effective means of reducing error than the removal of positional variance; the elimination of rows and columns has only reduced the variance from 10,024 to 7421 (Table IV), whereas the regression based on the total sums of squares (*i.e.* ignoring rows and columns) reduces it to 1754. This suggests that results of some value can be obtained with rubber where previous records are known, but where the arrangement of the plots does not permit elimination of the positional effect.

PRACTICAL CONCLUSIONS.

The results of this very brief and incomplete investigation show that the utilisation of previous crop records can be of considerable value in field experimentation with rubber. From a practical standpoint, also,

the method should be well suited to this crop. A trial of fertiliser treatments, for example, is a long-range investigation in which the results cannot be fully evaluated for some years, and the loss of time and increase in labour incurred in compiling one year's preliminary records are of small importance if the value of the subsequent experiment is to be materially enhanced. The figures show, however, that the advantage of the correction becomes gradually less marked as the gap between the "experimental" and "preliminary" years widens, and it is probable that the main value of the uniformity trial is in enabling the experimenter to draw sound preliminary conclusions, and to avoid false deductions, in the early years. This would more than counterbalance the loss of one year involved.

A consideration of practical details would be out of place in this paper. One point, however, must be mentioned. There is often a substantial difference in yield from the two sides of an individual tree, and it would seem to be important, therefore, that the uniformity trial should include equal tapping periods on the two panels. This is an easy matter under Ceylon conditions where an annual or six-monthly change of the panel is the usual practice, and it may be concluded that one year's records, with a "change-over" after six months, should prove a valuable precursor to any major field experiment with rubber in Ceylon.

SUMMARY.

1. Sanders' method of utilising previous crop records to correct experimental results by means of a linear regression is briefly described.

2. The method is applied to yield figures from rubber trees in Sumatra, and the precision of a dummy experiment is thereby increased nearly fourfold when the "preliminary" and "experimental" years are consecutive. When the "experimental" year is three years later than the "preliminary" year, the error variance of the adjusted yields is reduced to about a half.

3. It is concluded that not only has the method of correction been of value in the particular instance investigated, but that a uniformity trial utilised in this way should be of practical value in any major field experiments with rubber.

The writer is greatly indebted to Prof. R. A. Fisher, F.R.S. and Mr F. Yates of the Statistical Department, Rothamsted Experimental Station, who suggested this line of investigation and guided its progress

184 *Uniformity Trials in Field Experiments with Rubber*

throughout, and to Mr T. Eden, of the Tea Research Institute of Ceylon, who kindly read and criticised this paper.

REFERENCES.

- (1) SANDERS, H. G. *J. Agric. Sci.* (1930), **20**, 63.
- (2) EDEN, T. *J. Agric. Sci.* (1931), **21**, 565.
- (3) LORD, L. *Dept. of Agric. Ceylon Bull.* No. 82.
- (4) FISHER, R. A. *Statistical Methods for Research Workers* (4th edition), p. 252. Edinburgh, 1932.

(Received November 28th, 1933.)

OBSERVER'S BIAS IN SAMPLING-OBSERVATIONS ON WHEAT

F. YATES and D. J. WATSON

(Rothamsted Experimental Station)

IN a set of experiments conducted at a number of different places it is frequently necessary to employ a different observer for each experiment. In such cases the interchange of observers is usually impossible owing to the expense involved. Anything, therefore, in the nature of a bias appertaining to any particular observer will not appear in the experimental error. In certain types of observation such bias may assume considerable magnitude. Any opportunity of comparing different observers should therefore be seized on and made use of, especially as such opportunities are often rare.

In March of this year a conference of the observers of what are somewhat anomalously known as the Wheat Precision Observations was held at Rothamsted. This conference provided just such an opportunity, and enabled an experiment to be made to determine to what extent the counts by various observers differed from one another, and how far this difference was constant for a given observer and consequently in the nature of a bias. A brief account of the results of this experiment may be of general interest and will be given here.

The outline of the Wheat Precision Scheme is as follows: Sampling observations on wheat, which it is hoped will extend over a series of years, are now in progress under the supervision of the Agricultural Meteorological Committee, acting for the Ministry of Agriculture and Fisheries and the Department of Agriculture for Scotland and the Meteorological Office. These observations are designed to determine the principal events which mark the progress of the wheat plant from germination to maturity, so that the effect of weather conditions, in combination with varying soil types, can be studied. Observations are conducted at a number of centres, eight in 1932-3, and ten in the current season, scattered throughout the south and midlands (with the exception of one in Scotland). The principal events determined are: (1) appearance above ground, (2) tillering, (3) emergence of ears, (4) when fit for cutting; quantitative measurements of the density and growth of the crop, and the final yield, are also taken. A full account of the results of the first year's observations has been given elsewhere [1].

In order to determine the various dates and quantitative measurements with accuracy a considerable observational technique has been evolved. It is not proposed to describe the technique in full here, but it may be mentioned that the sampling unit, on which all observations except those for appearance above ground are taken, is a set of four contiguous quarter-metre rows. Thirty-two such samples are observed at each date for each variety, two from each half of each of the eight plots of that variety, the positioning within the half-plot being random. A new selection is made at each date.

The conference was planned to take place when observations were being made for tillering. Date of tillering is arbitrarily defined as the date at which the ratio of shoots (including the original shoot) to plants reaches the value of 2 : 1. It is determined by making counts for both plants and shoots at four weekly intervals, two before and two after this ratio has been reached, the actual date being ascertained by interpolation by means of the best fitting straight line. It will be realized that considerable judgement is required to determine what is in fact a separate plant, and what a tiller, particularly if the plants are crowded.

The actual test was planned for ten observers. Ten sampling units were marked off on a suitable piece of wheat and each of these sampling units was counted by all observers. The order of counting was determined by a Latin-square arrangement, so that each observer had a definite non-clashing order assigned to him.

Unfortunately the arrangement broke down in two particulars. Newport II observer mistook the instructions and observed his plots in the wrong order (the different rates of counting by the different observers explained why this mistake was not immediately discovered) and Rothamsted put forward a composite observer in the shape of three individuals. Fortunately neither of these errors has seriously disturbed the analysis of the results. Apart from these mistakes the observations were satisfactorily carried out. At the end of the experiment the plants were dug up by hand and plants and shoots of each quarter-metre counted.

TABLE I. *Discrepancies in Counts of Plants*

Observer	Sampling Unit										Total
	1	2	3	4	5	6	7	8	9	10	
Plumpton . . .	— 4 ¹	— 9 ⁴	— 2 ¹	— 0 ⁵	— 1 ⁷	— 10 ¹	+ 1 ¹⁰	— 4 ⁵	— 4 ⁹	+ 2 ³	— 31
Long Sutton . .	— 3 ⁶	— 10 ³	0 ⁹	— 4 ¹	0 ³	+ 2 ⁵	+ 2 ⁷	— 2 ¹⁰	— 9 ⁴	+ 0 ⁶	— 18
Cirencester . .	— 8 ⁹	— 18 ²	— 8 ³	— 5 ¹⁰	— 10 ¹	— 9 ²	— 11 ⁸	— 12 ⁴	— 21 ¹	— 6 ⁷	— 108
Boghall . . .	— 4 ¹	— 5 ⁴	0 ¹⁰	— 1 ¹	+ 2 ⁸	— 7 ³	+ 3 ⁹	— 8 ³	+ 5 ⁷	+ 0 ⁴	— 13
Newport II . . .	— 4 ¹⁰	— 8 ⁹	0 ⁵	+ 1 ⁴	+ 1 ¹	— 2 ³	+ 5 ³	— 1 ⁷	— 1 ²	+ 4 ⁵	— 1
Seale-Hayne . .	— 14 ¹⁰	— 22 ⁸	— 15 ⁹	— 16 ²	— 15 ¹	— 16 ⁷	— 19 ⁴	— 14 ⁹	— 30 ⁵	— 15 ³	— 176
Newport I . . .	— 4 ⁸	— 9 ¹⁰	— 2 ⁵	— 4 ²	0 ⁹	— 4 ¹	— 5 ¹	— 4 ⁷	— 0 ³	— 3 ⁴	— 35
Woburn . . .	— 10 ⁷	— 11 ¹	— 10 ⁴	— 6 ⁹	— 4 ³	— 11 ⁸	— 12 ⁵	— 7 ²	— 18 ⁸	— 6 ¹⁰	— 95
Wye . . .	— 17 ³	— 10 ¹	— 10 ¹	— 2 ⁷	— 2 ⁴	— 6 ¹⁰	— 8 ²	— 4 ⁸	— 17 ⁴	— 2 ⁹	— 78
Rothamsted . .	— 2 ⁴	— 6 ⁷	— 1 ²	+ 2 ³	+ 1 ⁹	— 1 ³	+ 2 ⁹	— 7 ¹	— 5 ¹⁰	+ 6 ⁵	— 11
Mean . . .	— 7	— 11	— 5	— 3	— 3	— 6	— 4	— 6	— 10	— 1	— 58
True Value . .	54	70	43	50	54	65	61	58	74	56	585

The small figures indicate the order of the observations.

Time Means

Time: 1	2	3	4	5	6	7	8	9	10
Mean: 50.2	52.0	52.4	51.9	52.6	52.0	54.7	54.1	54.5	54.0

Table I shows the discrepancies in plant-count of the individual observers. Inspection of the table immediately indicates that there is a strong negative bias in most of the observers, and that this bias differs from observer to observer and also from sample to sample. Inspection of the discrepancies of the individual quarter-metres (not shown here) shows, even more clearly than the totals of the samples, that this negative bias is most pronounced where the plants were most dense. This is of course

to be expected. It is noticeable, however, that the under-estimation of the more biased observers is not confined to the more congested samples and is not disproportionately great on such samples.

The mean discrepancies of times of observations 1 to 10 are shown at the foot of Table 1. In these means the discrepancies of Newport II are allotted to the times at which the observations should have been made, and not to the true times; the time means are thus estimates of time-differences unbiased by observer or sample differences; the fact that the times of Newport II were not the true ones will merely produce a slight watering down of the time effect, if any.

TABLE 2. *Analysis of Variance: Plant-counts*

	Degrees of freedom	Sum of squares	Mean square
Sampling units . .	9	818.64	90.96
Observers	9	2851.44	316.83
Times { Regression . .	1	140.12	140.12
{ Remainder . . .	8	46.52	5.82
Error	72	759.72	10.55
Total	99	4616.44	

The full analysis is given in Table 2. Time-effects are established as significant on considering the linear regression of the ten values. The observers themselves attributed this time-effect as the sorting out of the shoots by the earlier observers, so that the plants were more easily distinguishable. The fact that no such effect was observed on the shoots indicates that this is probably the true explanation.

It is not considered that the two mistakes already alluded to have seriously disturbed the analysis. The time-effect is not sufficiently large for the mistake of Newport II to produce appreciable bias in the sample or observer means and there appears to be little difference between the three Rothamsted observers.

TABLE 3. *Shoot-counts*

Observer:	Pl.	L.S.	Cir.	Bog.	N.II	S.H.	N.I	Wob.	Wye	Roth.
Count:	707	723	701	711	693	682	707	669	682	695
Mean: 697.					By digging: 755.					

The total shoot-counts of each observer is given in Table 3. Here also there is a serious under-estimation of the true value as determined by digging, but there is much less variation between the different observers. The under-estimation is here presumably due to the fact that some of the shoots counted on digging had not fully made their appearance above ground, though on the count by digging shoots that had clearly not made their appearance were not counted.

In the analysis of variance of shoot-counts (not reproduced) the differences between observers reach the 5 per cent. level of significance, but time of observation shows no effect. The residual variance, 10.0, closely approximates to that of the plant-counts.

Since the counts are taken with the object of determining the ratio of

shoots to plants it is of interest to see how far this ratio is affected by the counting errors. Inasmuch as the errors of counting may to a certain extent be compensating, low plant-counts being associated with low shoot-counts (though this does not occur to any great extent), the ratio for the various observers will be the quantity to examine. Table 4 shows

TABLE 4. *Ratio, Shoots: Plants*

Observer:	Pl.	L.S.	Cir.	Bog.	N.II	S.H.	N.I	Wob.	Wye	Roth.
Ratio:	1.28	1.28	1.47	1.24	1.19	1.67	1.29	1.37	1.35	1.21
Mean: 1.335					Estimated: 1.214					

this ratio. Assuming that there are 710 'visible' shoots the true ratio is 1.214. The mean ratio of all observers is 1.335, giving a mean bias of 0.121. The variance about this mean is 0.02063, giving 0.144 as the observer's standard error. The distribution is decidedly skew, half the observers obtaining ratios between 1.19 and 1.29 whereas the other half are all greater than 1.29, the worst being 1.67. It should be remembered, also, that the conditions of observing cause this bias to be underestimated, since all times have been included and it has been shown above that the bias was reduced as the observations progressed. In practice each sampling unit is observed once only. The mean ratio of the first set of observations, 1.39, may consequently be regarded as a better value from which to estimate the mean bias. This gives a mean bias of 0.18, nearly twice the value obtained above.

It is, of course, doubtful how far the bias of any particular observer remains constant. Probably it is fairly constant for plants and shoots of a given density on the same soil type, but it may quite probably increase very considerably as the density of shoots increases. The conditions of the experiment in one respect were particularly easy, as the ratio of shoots to plants was still low. The plant number, 1,872 per 32 metres, on the other hand, was high, compared with last year's values, which at tillering ranged from 368 to 1,578 per 32 metres. This was partly because thinly populated areas were avoided in selecting the samples for observation.

The average rate of tillering last year was about 35 tillers per 100 plants per week. Assuming that the bias of each observer is constant this would indicate that the bias of the worst observer introduced an error of about nine days in the date of tillering, whereas the best observers had little or no bias. Since the last four stations to tiller last year tillered within an interval of a fortnight, errors of this magnitude cannot be regarded as by any means negligible. It is hoped, however, that a few actual counts by observers on subsequently dug samples will serve to eliminate such large bias as that of the observer from Seale-Hayne. As a result of this experiment this practice is now to be explicitly recommended.

REFERENCE

1. Wheat Precision Observations. J. Min. Agric. 1933, 39, No. 12, 1082-4; 40, No. 3, 206-8; No. 7, 591-3; No. 10, 903-6.

(Received March 27, 1934)

THE BORDER EFFECT IN PLOT EXPERIMENTS

A. V. COOMBS

(*Statistical Department, Rothamsted Experimental Station, Harpenden, Herts.*)

Introduction

IN field experiments, particularly manurial trials, the influence of the more favourable treatments on the edge rows of those less favourable has long been apparent. But not much importance has been attached to it in the past, for the plots were comparatively large and square in shape, so that the edge rows only accounted for a small fraction of the area of the plot, and hence there was little cause for discarding them. Although there may have been border effects, the ratio the edge rows bore to the plot was so small that the results would show little or no appreciable difference whether the edge rows were discarded or not. However, since the introduction of the narrow-and-small-plot methods of experimentation, in which discarding the edge rows may sometimes mean rejecting almost half the material, there has been a temptation to include the edge rows. To the practical field experimenter, whose chief anxiety is to keep the standard error as low as possible, discarding may appear not only a waste of good material, but detrimental to the efficiency of the experiment. It also involves extra labour at harvest.

The object of this paper is to illustrate the inadvisability of including the edge rows in variety trials and cultivation experiments with narrow or very small plots. It will be shown that the rejection of the edge rows in these experiments has no deleterious effects on the efficiency of the experiments, and that there are border effects attributable to competition and to the trespassing of the treatments, which, when the entire plot is harvested, may result in a considerably biased estimation of the treatment differences.

In manurial trials the rejection of edge rows involves a further factor, since the relative yields of edge rows and centre rows will be affected by any uneven distribution of the manures. The advisability of rejecting edge rows is thus considerably more doubtful, and it appears that special precautions should be taken in experiments with small or narrow plots to ensure that the edge rows receive their proper share of the manures.

Material

The only available data for this study were those from four experiments: a manurial experiment with kale conducted at Woburn Experimental Station, a cultivation and thinning experiment with kale conducted at Rothamsted, and two sugar-beet experiments (one spacing and manurial, and one manurial) also conducted at Rothamsted. In Expt. I the weight of each row was recorded separately. In Expt. II each plant was weighed separately. In Expt. III the two edge rows were weighed together. In Expt. IV the edge rows and end plants of the other rows

were weighed together. In the two sugar-beet experiments only the roots have been considered in this study. A summary of the main treatment yields with and without the rejection of the edge rows, and other particulars of each experiment are given in Tables 2. In Expts. II and IV the edge rows and end plants of the other rows have been discarded, and in Expts. I and III the edge rows only have been rejected. The area of the plots with and without the rejection of the edge rows, the number of rows, and the ratio the edge rows bear to the plots are given in Table 1.

TABLE 1

<i>Experiment</i>	<i>I</i>	<i>II</i>	<i>III</i>	<i>IV</i>
Area without rejection (acres) . . .	0.025	0.0046	0.018	0.0040
Area with rejection (acres) . . .	0.015	0.0025	0.014*	0.0029
Number of rows per plot . . .	5	5	8*	12
Approximate fraction of plot discarded	$\frac{2}{5}$	$\frac{1}{2}$	$\frac{1}{4}$ *	$\frac{2}{7}$

* Where rows are spaced 15 in. apart.

The dimensions of the plots are: Expt. I, 109 ft. rows \times 10 ft.; Expt. II, 20 ft. rows \times 10 ft.; Expt. III, 80 ft. rows \times 10 ft.; and Expt. IV, 11 ft. 8 in. rows \times 15 ft.

TABLE 2. *Summaries: Edge Rows rejected and included*

Experiment I. Marrow-stem Kale, Rothamsted, 1932.

System of replication: 6 randomized blocks of 4 plots each.

Treatments: Unthinned; Thinned to 18 in., in combination with ordinary cultivation (June 2-4, horse-hoe); intensive cultivation (June 2-4, horse-hoe, July 4, 19, 27, motor-hoe).

Basal manuring, per acre: 16 tons dung, 2 cwt. sulphate of ammonia, 3 cwt. superphosphate, and 3 cwt. 30 per cent. potash manure salt.

Green Material

<i>Tons per acre</i>	<i>Unthinned</i>		<i>Thinned</i>		<i>Mean</i>	
	<i>Rejected</i>	<i>Included</i>	<i>Rejected</i>	<i>Included</i>	<i>Rejected</i>	<i>Included</i>
Ordinary cultivation . . .	27.65	28.02	25.18	23.90	26.42	25.96
Intensive cultivation . . .	25.51	27.92	23.63	23.54	24.57	25.73
<i>Mean</i> . . .	26.58	27.97	24.40	23.72	25.50	25.84

Standard errors: 0.323 tons (rejected), 0.366 tons (included).

Experiment II. Marrow-stem Kale, Woburn, 1932.

System of replication: 8 \times 8 Latin square.

Basal manures (plots receiving no dung): 0.5 cwt. P_2O_5 per acre as superphosphate, and 30 per cent. potash manure salts at the rate of 1 cwt. K_2O per acre.

Green Material

<i>Tons per acre</i>	<i>No S/Amm.</i>		<i>S/Amm. 0.2 cwt. N</i>		<i>S/Amm. 0.4 cwt. N</i>		<i>S/Amm. 0.8 cwt. N</i>		<i>Mean</i>	
	<i>Rejected</i>	<i>Included</i>	<i>Rejected</i>	<i>Included</i>	<i>Rejected</i>	<i>Included</i>	<i>Rejected</i>	<i>Included</i>	<i>Rejected</i>	<i>Included</i>
No dung	12.58	13.29	17.64	17.76	20.35	19.67	26.15	24.36	19.18	18.77
Dung 15 tons	20.28	19.19	22.87	21.24	24.58	23.07	30.56	28.74	24.57	23.21
<i>Mean</i>	16.43	16.24	20.26	19.50	22.46	21.67	28.36	26.55	21.88	20.99

Standard errors: 0.863 tons (rejected), 0.713 tons (included).

Experiment III. Sugar-beet, Rothamsted, 1933.

System of replication: 12 randomized blocks of 6 plots each.

Certain degrees of freedom representing interactions are confounded with block differences. Absolute replication if fourfold.

Treatments: 0.0, 0.3, 0.6 cwt. N per acre given as sulphate of ammonia in combination with rows spaced 10, 15, 20 in. apart.

Basal minerals: (0.5 cwt. P_2O_5 per acre as superphosphate, 1 cwt.

K_2O per acre as 30 per cent. potash manure salt) ploughed under and harrowed in.

Roots (washed)

Mean of all levels of N

<i>Tons per acre Edge Rows</i>	<i>Rows spaced 10 in. apart</i>		<i>Rows spaced 15 in. apart</i>		<i>Rows spaced 20 in. apart</i>		<i>Mean</i>	
	<i>Rejected</i>	<i>Included</i>	<i>Rejected</i>	<i>Included</i>	<i>Rejected</i>	<i>Included</i>	<i>Rejected</i>	<i>Included</i>
Basal minerals ploughed in	8.12	8.05	7.04	6.93	5.77	5.73	6.98	6.90
Basal minerals har- rowed in	7.22	7.28	6.07	5.99	4.99	4.98	6.00	6.08
<i>Mean</i>	7.67	7.66	6.56	6.46	5.38	5.36	6.54	6.49

Standard errors: 0.14 tons (rejected), 0.13 tons (included).

Experiment IV. Sugar-beet, Rothamsted, 1933.

System of replication: 3 randomized blocks of 16 plots each.

Treatments: (1) 0.0 and 0.6 cwt. N per acre as sulphate of ammonia applied on the surface; (2) No PK, PK applied in the surface soil, PK applied in subsoil, PK applied in surface and subsoil (double dressing); ($P = 0.5$ cwt. P_2O_5 per acre as superphosphate. $K = 1.0$ cwt. K_2O per acre as 30 per cent. potash manure salt); (3) No dung, 20 tons dung, applied in surface soil, subsoil, and in surface and subsoil (double dressing).

Roots (washed)

<i>Tons per acre</i>		<i>PK only</i>			<i>PK and dung</i>			<i>Mean</i>
		<i>No PK or dung</i>	<i>Shallow</i>	<i>Deep</i>	<i>Shallow and deep</i>	<i>Shallow</i>	<i>Deep</i>	
Edge rows rejected	No N	4.82	4.99	6.37	6.50	7.88	7.74	6.62
	N	4.76	5.43	6.64	6.68	7.52	7.30	6.76
	<i>Mean</i>	4.79	5.21	6.51	6.59	7.70	7.52	6.69
Edge rows included	No N	4.85	4.80	6.04	6.29	7.57	7.57	6.36
	N	4.81	5.34	6.46	6.55	7.15	6.96	6.53
	<i>Mean</i>	4.83	5.07	6.25	6.42	7.36	7.26	6.45

Standard errors: 0.650 tons (rejected), 0.650 tons (included).

Standard Errors

The two main sources of error in plot experiments are variations due to soil heterogeneity and variations due to the genetical composition of the plant. As we reduce the size of the plot we should reduce the error due to broad irregularities of soil, but as the number of plants is reduced the error due to plant variation and local soil irregularities is liable to be increased. Variations in the edge rows due to the trespassing of the treatments or to similar causes will be an additional source of error, and provided there are sufficient plants, discarding the edge rows may increase the efficiency of the experiment.

Each experiment has been analysed by the procedure of the analysis of variance, both with the edge rows rejected and with the edge rows included, and the standard errors have been calculated in each case. The standard errors are given in Table 3. In general the rejection of the edge rows has made practically no difference to the standard errors. In Expt. I the rejection of the edge rows has reduced the standard error per plot from 3.52 per cent. to 3.10 per cent. Even in Expt. II, in which the rejection of the edge rows and end plants of the other rows has reduced the number of plants from 100 to 54, the standard error has shown only a slight increase.

It may be concluded that, in general, discarding the edge rows will have no deleterious effects on the efficiency of the experiment, and that in certain instances a reduction in the standard error is to be expected.

TABLE 3

<i>Experiment</i>	<i>Percentage standard error per plot</i>				<i>No. of plants per plot</i>			
	<i>I</i>	<i>II</i>	<i>III</i>	<i>IV</i>	<i>I</i>	<i>II</i>	<i>III</i>	<i>IV</i>
Edge rows included .	3.52	9.60	7.21	17.70	360*	100	760†	168
Edge rows rejected .	3.10	11.16	7.65	17.53	216*	54	570†	120

* Where plots are thinned.

† Where rows are spaced 15 in. apart.

Bias

In field experiments where the size and shape of the plots are such that the edge rows and edge plants comprise a comparatively large proportion of the plot area, any edge-row effects will make an appreciable difference between the estimation of the yield from the entire plot and that from the plot with the edge rows rejected. For example, the edge-row effect may have been due to the trespassing of the treatments, so that the edge rows of the untreated plots may have benefited by being adjacent to the treated plots. The estimated yield from the untreated plots with the edge rows included will be higher than that from the plots with the edge rows discarded. Thus the increase in yield due to the application of the favourable treatment will be less in the former case than in the latter. It will therefore be apparent that the more unbiased

estimate of the treatment yields will be that from the plots with the edge rows discarded, for there will be a bias in favour of the untreated plots and against the treated plots when the entire plot is harvested.

Both positive and negative biases are possible. They may arise in several ways.

Bias due to plant competition.—In thinning and spacing experiments the edge rows of the favourably treated plots when adjacent to the unfavourably treated plots are likely to produce higher yields than the central rows, and the edge rows of the unfavourably treated plots lower yields than their inside rows. The bias of the thinning effect in Expt. I is a striking example of the magnitude such competition effects may attain.

Bias due to cultivation.—When two adjoining plots each receive different 'after seeding' cultural treatments, the problem always arises as to what cultivation the space between the edge rows should receive. Is it to receive one or other of the cultivations? Or is it to be left uncultivated? Whatever treatment it receives, the yields of the edge rows are liable to differ from those of the central rows. This is illustrated in the cultivation treatment of Expt. I, where there was actually a statistically significant (but inexplicable) reversal of cultivation effects on the edge rows.

Bias due to manurial treatments.—This bias will be due primarily to the trespassing of roots and manures. The trespassing of manures may occur in two ways. There may be the actual surface straying of the manures due to their inaccurate application. This is more likely to occur with artificial fertilizers than with farm-yard manure, for any straying in the application of the latter will be visible and the particles that have strayed can easily be removed. The other form of trespassing is the lateral spreading of the manures under the surface of the soil, because all the manure will not permeate into the soil in a strictly vertical direction; although the manures may have been applied with great care and accuracy, there will still be a trespassing of the manures on to the adjoining plot. This trespassing will be greater at the lower depths of the soil. Thus it should be more apparent with deep-rooted than with shallow-rooted plants.

Bias due to uneven fertilizer-distribution.—To obtain even distribution of manures in small-scale experiments, the experimenter usually relies entirely on the skill of the man applying them. If the edges of the plot are over- or under-dosed, the amount applied to the insides of the plot will be respectively less or more. In such cases the rejection of edge rows may give more biased results than their retention. Distribution errors of this type may be expected to reveal themselves by producing consistently lower (or higher) yields on the edge rows of the manured plots with little or no difference on the unmanured plots. Unfortunately, however, there are other causes, e.g. excessive trampling, which tend to lower the yields of the edge rows, and such effects in combination with the trespassing effects already discussed will produce very similar results to those that would be obtained by giving an undue share of the manures to the centre of the plot.

That there are considerable differences between the apparent responses

to treatments according as the edge rows are included or excluded, will be seen from Table 4, where the difference between the increase in yield from the plots with the edge rows discarded and that from the plots with the edge rows included is expressed as a percentage of the increase in yield from the former. For example, in Expt. I the difference between the increases due not to thinning is 2.07 tons, which when expressed as a percentage of 2.18 tons gives a difference of 95 per cent. For non-manurial treatments these percentage differences may be regarded as estimates of the bias caused by the retention of the edge rows.

The results of Expts. II and IV given in Table 2 suggest that there was some unequal spreading of the manures in addition to trespassing. This point is examined in more detail in the next section.

TABLE 4

Experiment	Treatment	Increase with edge rows discarded	Increase with edge rows included	Per- centage difference
Tons per acre				
I. Kale	Unthinned <i>v.</i> thinned	2.18	4.25	+ 95
	Ordinary cultivation <i>v.</i> intensive cultivation	1.85	0.23	- 88
II. Kale	0.8 cwt. N as S/A <i>v.</i> no N	11.93	10.37	- 14
	15 tons dung <i>v.</i> no dung	5.39	4.44	- 18
III. Sugar-beet (roots)	Basal minerals ploughed under <i>v.</i> basal minerals harrowed in	0.89	0.82	- 8
	Rows spaced 10 in. apart <i>v.</i> rows spaced 20 in. apart	2.29	2.30	0
IV. Sugar-beet (roots)	2 units PK <i>v.</i> no PK	1.79	1.60	- 11
	40 tons dung <i>v.</i> no dung	1.95	1.51	- 23

Note. The increase is in favour of the first-mentioned treatment in each case.

Estimation of the Magnitude of the Edge Effects

The bias introduced into a treatment difference does not give a direct estimation of the trespassing or competition effect, since the magnitude of the bias depends also on the actual experimental arrangement, and the size and shape of the plots. A direct and much more accurate estimate can be obtained by utilizing the yields of the individual edge rows.

If an edge row of a plot receiving treatment *A* is contiguous to a plot receiving treatment *B*, and t_a and t_b represent the increase (per row) produced by treatments *A* and *B* when there is no edge effect, the yield of the edge row may be expected to be increased by an amount

$$\lambda t_a + \mu t_b,$$

where $\lambda + \mu = 1$. Assuming for the moment that t_a and t_b are known and their difference is δt , the difference between the yield of the edge row, y_e , and the mean yield of the central rows, y_c , may be expected to be given by

$$\begin{aligned} y_e - y_c &= (\lambda t_a + \mu t_b) - t_a \\ &= \mu(t_b - t_a) \\ &= \mu \delta t, \end{aligned}$$

subject to errors of various types. In this equation the effect of any fertility or other difference affecting the whole of the plot is eliminated. There will be one such equation for every edge row except those at the edges of the experimental area.

If each plot is subjected simultaneously to two different treatments, as in a complex experiment, and the effects of these treatments are assumed to be additive, the equation will become

$$y_e - y_c = \mu_1 \delta t_1 + \mu_2 \delta t_2.$$

When both edge rows of the plot are weighed together, as happened in Expt. III, the appropriate equations for the two edge rows separately must be combined into a single equation of the type

$$y_e + y'_e - 2y_c = \mu_1(\delta t_1 + \delta t'_1) + \mu_2(\delta t_2 + \delta t'_2).$$

Estimates of the values of the edge-effect coefficients μ_1 and μ_2 may be made by the method of least squares, i.e. the method ordinarily used for partial regressions and fitted constants.

The procedure will be approximate in two respects. First, we do not know the true values of the treatment differences, and estimates of them must be made from the experimental results (using the yields with the edge rows rejected). If the soil fertility varies continuously, it is not difficult to see that this will tend to exaggerate (in a positive direction) the values of μ obtained. If, however, the treatment effects are large compared with their standard errors, and the experiment is also large, so that the average treatment differences are well determined, this disturbance should not be great, but clearly it would be inadvisable to attempt to take into account the interactions between the different types of treatment.

The second source of disturbance is due to the fact that the same centre rows occur in two equations (one for each edge row of the plot), and the errors will therefore not be wholly independent. This will tend to exaggerate the significance of the results when tested in the ordinary manner. It could be avoided by combining the two equations for each plot into one (as was necessarily done in Expt. III, where both the edge rows were weighed together) but this would sacrifice a considerable part of the available information. Alternatively differences of each edge row with the next neighbouring row might have been taken, but even here some correlation is likely to remain, and the errors would probably be larger.

The possibility of unequal distribution of the manures, and the effects of trampling, &c., on the edge rows, remain to be considered. As far as trampling is concerned it will be reasonable to assume that the yields of the edge rows are diminished by a constant amount. Unequal distribution may be represented as follows. If the true density of the manures per unit area is ρ , and the density on the edge rows is $\rho(1-\lambda)$, the density on the centre rows of, say, a five-row experiment will be $\rho(1+\frac{2}{3}\lambda)$, and therefore the difference in yield between the edge rows and the centre rows may be expected to be

$$-\frac{2}{3}\lambda t,$$

where the response (assumed linear) to the correct dressing is t and spreading and competition effects are neglected.

Expts. II and IV are the only experiments involving direct responses to manures. Expt. IV, however, was not very suitable for the application of the method, as the whole of the edge of each plot was weighed together. Expt. II, when analysed by this method, gave some rather surprising results. Using a regression equation of the type

$$y_e - y_c = c + \mu_N \delta t_N + \mu_D \delta t_D - \frac{5}{3} \lambda_N t_N - \frac{5}{3} \lambda_D t_D,$$

where N indicates sulphate of ammonia, and D dung, the following values for the coefficients were obtained.

$$\begin{aligned} c &= -0.71854 \pm 1.552 \\ \mu_N &= +0.11176 \pm 0.077 \\ \mu_D &= -0.18940 \pm 0.119 \\ \lambda_N &= +0.06300 \pm 0.075 \\ \lambda_D &= +0.24121 \pm 0.111 \end{aligned}$$

Only λ_D is significant, but its actual value appears to be unduly high. It is scarcely likely that the edge rows of the dunged plots received only about three-quarters of their true dressing; on the other hand λ_N and λ_D are not significantly different, and their mean value, 0.15, indicating about five-sixths of the proper dressing on the edge rows, is not unreasonable.

Though neither of the coefficients of trespassing μ_N and μ_D is significant, the negative value of μ_D is in itself a little surprising. It may be that this is a competition effect, but some fortuitous interaction between μ_D and λ_D may possibly account for these anomalies; for instance if λ_D were assumed equal to 0.1, this would automatically reduce μ_D considerably.

In Expt. II, therefore, it appears that the main cause of the observed bias is the unequal distribution of the manures. It should be remembered, however, that the effects of trespassing and unequal distribution are somewhat similar (especially when as here a constant term c is included in the regression equation), and it would be unwise to conclude on the evidence of this experiment alone that trespassing effects of manures are necessarily always negligible.

Expt. I, in which there are no manurial treatments, shows very striking edge effects. A slightly different form of equation was used for the reason that the space between intensively cultivated and ordinarily cultivated plots was given ordinary cultivation. The equation for the edge row of the intensively cultivated plot must therefore contain a μ for cultivation effect, whilst the equation for the edge row of the ordinarily cultivated plot will contain no μ for cultivation effect, since both sides of this row receive ordinary cultivation. The expected value of this μ will be 0.5. The calculated value, $+0.95 \pm 0.637$, is considerably, but not significantly, greater.

The thinning in this experiment shows a very remarkable competition effect, the outside rows of the unthinned plots growing exceptionally well owing to the reduction of competition from their thinned neighbours, which in their turn were unduly depressed. The value of μ , -1.58

± 0.308 , indicates that the difference between the thinned and unthinned edge rows was no less than four times the difference between the thinned and unthinned central rows.

In Expt. III it is not possible to consider inequalities of distribution, since there are no plots without basal manures, and therefore their effect is unknown. Nitrogen showed no effects and was therefore omitted. The value of μ for 'basals', $+0.24 \pm 0.121$, is significant. The value of μ for spacing, $+0.14 \pm 0.064$, is also significant, and not quite significantly different from the value of 0.25, which would be expected if there were no competition effects.

In only one of the three experiments did the value of c approach significance, thus indicating that the edge rows did not suffer greatly from extra treading, &c.

Summary

1. The results of four experiments in which the edge rows were harvested separately were examined in order to determine the importance or otherwise of rejecting edge rows in experiments having small or narrow plots.

2. It was found that the rejection of edge rows did not appreciably increase the standard errors of the experiments.

3. It is shown that in experiments where the plots are narrow or very small, the retention of the edge rows will give decidedly biased estimates of the treatment differences, due to the trespassing and competition effects of the treatments on the edge rows. It is also shown that in manurial experiments the discarding of edge rows may give more biased results than their retention, due to the unequal distribution of the manures between the centres and outsides of the plots.

In conclusion, I wish to acknowledge my indebtedness to Mr. F. Yates, Head of the Statistical Department, Rothamsted, under whose guidance this work has been carried out.

(Received June 5, 1934)

A COMPLEX PIG-FEEDING EXPERIMENT.

By F. YATES, B.A.

(Chief Statistician, Rothamsted Experimental Station,
Harpenden, Herts.)

(With One Graph.)

INTRODUCTION.

IN the last ten years there has been something of a revolution in agricultural experimental technique, as far as field crops are concerned, but it is only lately that the new methods have begun to spread to live-stock experimentation. It is likely that a similar improvement will be witnessed here also, both in the efficiency of experimentation, and in the validity of the results obtained, but there are many problems which require investigation before a technique which is not only logically and statistically sound but also reasonably efficient can be evolved.

The farm of the Rothamsted Experimental Station has for the last few years bred pigs fairly extensively. This activity was undertaken largely for commercial reasons in order to make efficient utilisation of products of the arable part of the farm which is necessary for the main experimental work of the station. It has, however, been the practice to record the growth of these pigs, and the Farm Director has, for his own information, from time to time undertaken a few simple experiments. The opportunity was thus to hand of making investigations in problems of animal husbandry and management at little cost, and in the summer of 1933 an experiment was started with a view to utilising the available material and contributing towards the solution of problems of experimental technique.

The animal nutrition station at Cambridge has for some time been working in the same field, and a paper by Dunlop has recently been published in this *Journal*(2), which gives an account of the methods adopted by that station and also contains an able review of previous work by other stations, both in this country and abroad. The Cambridge work, which was undertaken independently of that at Rothamsted, forms a most interesting parallel, and comparison of the similarities and differences of

the methods adopted at the two stations will serve to bring out the experimental problems involved.

In this paper we propose to review the first Rothamsted experiment, which is now completed, and then to make a comparison of the methods adopted with those of Cambridge. Finally a short discussion of possible lines of improvement will be given.

REQUIREMENTS OF A GOOD EXPERIMENTAL TECHNIQUE.

The first and perhaps the most important requirement of any method of experimentation is that the experiments should be so designed and executed that it is possible to draw sound inferences from the results. This is one of the objects of replication and the sole object of randomisation in experiments on field crops, and similar processes must be adopted in other types of experiment if the inferences are to escape criticism of a type which is difficult to refute. An animal husbandry experiment, for example, in which all the animals on one treatment are confined to one pen, and all the animals on the other to another pen, is always open to the objection that the pen of the less successful treatment was less favourably placed, or that the animals of this pen developed some disease, and the experimenter can only express his personal opinion that this was not in fact the case. Statistically we say that a valid estimate of error is required, that is, an estimate which is unbiased and includes all sources of error which affect the results, *and no others*.

The experiment described in this paper itself provides a striking illustration of the paramount importance of this requirement. Here many of the pigs without green food (one of the experimental treatments) became sick. There is no doubt that this sickness, whatever it was, was conditioned by lack of green food, but had the pigs without green food all been run in one pen or group of pens it is very probable that the sickness would have been attributed to extraneous causes, and its association with green-food deficiency overlooked, especially in view of the fact that previous experiments on green food at Rothamsted had indicated that pigs did not require this addition to their diet. In any case the conclusions drawn would have been subject to a very large element of doubt, and would by themselves have been of little or no value.

The second requirement is that the inferential basis of the results should be as wide as possible, that is, as many as possible of the types of material and combinations of circumstances over which the conclusions are intended to apply should be included. Thus a conclusion as to the nutrient value of a certain feeding stuff is definitely strengthened if

more than one breed and other factors such as amount of exercise are included in the experiment. This is one of the arguments in favour of the complex type of experiment which is proving so useful in agricultural field experimentation.

Thirdly, the technique must be efficient, that is, it must make the best use of the material and labour available. The complex type of experiment stands out as one of the most powerful methods of increasing efficiency.

Fourthly, the results must be capable of analysis by the available statistical methods. Improvement in statistical methods often suggests radical changes of design. For many purposes also it is important that the results should be capable of easy interpretation by those unversed in statistical procedure; this consideration sometimes, though perhaps not so often as is thought, conflicts with the requirements of efficiency.

It will be obvious, to those acquainted with the parallel fields of agricultural experimental technique on crops and live stock, that the methods of the former far surpass those of the latter when judged on the above standards. The complex type of experiment has scarcely as yet been applied to live stock, and the great majority of live-stock experiments provide an estimate of error which is of doubtful value.

The chief difference between crop and live-stock experiments which might account for this is the mobility in the material of the latter. A treatment, whatever its nature, once applied to a plot, only affects the plants on that plot, but in any feeding trial with animals different feeding can only be given by separating the animals at feeding time. This offers a strong inducement to run all animals on the same food in the same pen. Such communal feeding not only precludes any valid estimate of error but also prevents any wide variety of different feedings, since the number of these is limited by the number of available pens.

The obvious solution is individual feeding. Such feeding has the additional advantage of enabling the food consumption of each animal to be recorded. It is also likely to induce a more even growth by eliminating competition at feeding time; though such elimination, inasmuch as it departs from ordinary practice, cannot be regarded as wholly advantageous.

The difficulty of individual feeding varies widely according to the live stock to be experimented on, and the type of experiment. With animals fed indoors there is only the problem of providing suitable pens. Grazing experiments, on the other hand, present almost insuperable difficulties.

In one other respect most experiments with animals differ greatly from experiments with annual field crops. In experiments with animals the weight of any particular animal at the beginning of the experiment usually provides a valuable measure of the potential development of that animal.

The methods of utilisation of this information (or the parallel information of previous cropping in a perennial crop) provide an excellent illustration of the way in which improvement in statistical method may radically affect the design. Prior to the introduction of the analysis of covariance it was customary either to balance out the weights, *i.e.* assign animals as nearly as might be of equal total weight to each treatment, or group animals of nearly equal weight together and assign one or more from each group to each treatment. The former method makes any estimate of error impossible (except by an analysis of covariance), while the latter method, though useful and capable of yielding statistically valid results, introduces another set of restrictions into the design, and becomes progressively less efficient as the number of treatments is increased. The analysis of covariance enables the effects of differences in initial weight to be eliminated without any account being taken of them in the initial design. It thus gives greater freedom of design which can be utilised for other purposes.

THE ROTHAMSTED EXPERIMENT.

The Rothamsted experiment was designed to test the performance of pigs from weaning to bacon weight when fed on dry and on wet meal, with or without the addition of green food. The effect of varying the numbers in a pen was also tested, equal floor space being assigned to each pig.

Table I shows the arrangement. The experiment was in three blocks. Each block contained twenty-four pigs, six from each of four litters, with as far as was possible equal numbers of hogs and gilts from each. Each block was subdivided into three, to give one pen of eight, two pens of four, and four pens of two, the position of each sub-block within the block being determined by random choice. In each sub-block there were two pigs on each of the four feeding treatments. Sex and litter were equalised as far as possible over the various treatments, pigs of given sex and litter being allotted to their assigned treatments at random.

The composition of the meal is shown in Table II. This was varied from time to time to suit the needs of the growing pig. The introduction

of bran was made on account of the low quality of the middlings available during the latter half of the experiment.

The pigs were weighed weekly, before the afternoon feed. The food consumption was also recorded weekly, each pig being fed from its own bag. Feeding was *ad lib.*¹ twice daily, 7 a.m. and 3 p.m. Any small amounts of wet food left unconsumed at the end of the week were weighed and discarded. Dry food was returned to the bags.

Table I. *Arrangement of experiment.*

Block and duration ...	Block I (21 weeks)				Block II (22 weeks)				Block III (20 weeks)			
	9	12	19	21	17	20	29	48	27	28	35	58
Litter No. ...	7-9	10-9	13-6	12-0	9-7	11-7	11-3	12-1	8-1	10-4	10-7	12-6
Age at start (weeks) ...												
Sex ...	H G	H G	H G	H G	H G	H G	H G	H G	H G	All H	All G	H G
Dry and green food	8 2	2 8	. 4	. 4	8 .	4 2	. 8	2 4	. 2	8 4	8 4	2 .
Wet and green food	. 4	4 .	2 8	8 2	2 4	. 8	4 2	8 .	4 8	2	2	8 4
Dry food	4 .	. 4	2 8	8 2	2 4	. 8	4 2	8 .	4 8	2	2	8 4
Wet food	8 2	2 8	. 4	. 4	. 8	4 2	8 .	2 4	. 2	8 4	8 4	2 .

The number 2, 4 or 8, indicates that the pig was one of a pen of 2, 4 or 8 respectively.

H denotes hog (*i.e.* castrated male), G denotes gilt (*i.e.* female).

Table II. *Composition of feeding rations.*

Block	I ...	Weeks of experiment			
		1-3	4	5-18	19-21
"	II ...	1-3	4	5-14	15-22
"	III ...	1-3	4	5-9	10-20
Percentage rations					
Middlings		60	50	40	28
Bran		—	—	—	14
Hominy chop		—	15	20	18
Barley meal		20	25	30	30
Flaked maize		10	—	—	—
Fish meal		10	10	—	—
Meat meal		—	—	10	10

Two per cent. minerals (3 parts lime, 1 part salt) added to each ration.

Green food (kale, wheat, oats and vetches) fed twice daily at the rate of about $\frac{1}{4}$ lb. per head per day.

The pens were stoutly constructed of 1 in. deal boarding, with partitions 2 ft. 6 in. high. The dimensions of the pens (excluding the feeding pens) were as follows:

Pens of eight: 13 ft. \times 6 ft. 3 in.

Pens of four: 6 ft. 6 in. \times 6 ft. 3 in.

Pens of two: 3 ft. 3 in. \times 6 ft. 3 in.

¹ This expression, as here used, does not denote constant access to the food supply at all times, but merely unlimited food supply at feeding times. Similarly *rationing* is used to denote restricted food supply at feeding times.

The feeding pens were 1 ft. 8 in. \times 3 ft. 7 in. They were fitted with drop doors and each contained a small zinc feeding trough. One side of each feeding pen was of chain link netting, so that the pig could see its neighbour feeding. One of each four pens was arranged as an entrance to the main pen. Water was available in one of the feeding pens except at feeding time. The feeding pens were contiguous to the gangway between the blocks in the same manner as the Cambridge lay-out. The floor was concreted.

The pens occupied a corner of a large building whose two adjacent sides were of corrugated iron. There was a loft above, but in spite of this the temperature conditions were very variable, and during the hot spell of the summer of 1933 it was thought that the pigs were doing badly owing to the heat. Subsequent examination of the records, however, revealed that only the pigs without green food were affected.

The pigs themselves were not all the same breeding. One litter in each of the first and third blocks were pure Wessex, and the rest were by a Large White boar out of either a Wessex or Large Black sow.

METHOD OF ANALYSIS APPROPRIATE TO COMPLETE RESULTS.

We now propose to discuss the methods of analysis appropriate to the design adopted. The design at first sight appears somewhat complex, but from what follows it will be seen that with a full set of data the analysis is extremely simple.

Each block is complete in itself, in the sense that it is equalised as far as possible for sex and litter. An error can be computed for each block separately. Examining block I in detail, we observe that as many pigs of each litter and of each sex receive dry food as wet food; the same holds for green food, and for the numbers in a pen. The interaction between wet (W) and dry (D) feeding and green food (G), however, is not equalised for litters. If it were equalised it would be computed from the expression

$$Q = \frac{1}{8} S (WG - DG - W + D),$$

and this has two pigs of litters 19 and 21 in excess and two litters of 9 and 12 in defect. This expression therefore needs a correction for litter effects. The exact form of the correction can be obtained by fitting constants; if $S(L)$ represents a litter total the corrected estimate is found to be

$$Q' = \frac{1}{16} [S(WG - DG - W + D) + \frac{1}{3} S(L_9) + \frac{1}{3} S(L_{12}) - \frac{1}{3} S(L_{19}) - \frac{1}{3} S(L_{21})].$$

It is obvious by inspection that the litter effect is now eliminated.

In the analysis of variance the sum of squares appropriate for testing

this interaction will be $\frac{1}{3} Q'^2$, since the variance of Q' is $\frac{3}{4}$ that of a single pig. The estimate of error variance may be obtained by deducting from the total sum of squares the sum of squares for litter (estimated as usual from the litter totals), sex, numbers in pen, wet *v.* dry food, green food and the interaction of these last (calculated from the above formula). Note that since litters are partially confounded with the treatment interaction the litter sum of squares here obtained is not the appropriate one for testing litter effects.

There is left in error the interactions of numbers in pen with the feeding treatments. The two main interactions could if desired be taken out in the ordinary manner. The second order interaction, however, is partially confounded with litters; its extraction from error would therefore be more difficult. The above estimate of error also involves the assumption that the sex difference is substantially constant from litter to litter, so that the interaction of sex and litters may be considered as pure error. It will also be realised that numbers in pen is analogous to sub-blocks, and the ordinary experimental error is therefore not appropriate for numbers in pen comparisons.

Blocks II and III are similar in design to block I with the exception that in neither case were four litters with three hogs and three gilts available. In the arrangement adopted sex is no longer orthogonal with litters (but is still orthogonal with treatments). The analysis is again quite simple. The sum of squares for sex for block II is given by

$$\frac{3}{8} \{S(H) - S(G) + \frac{1}{3} S(L_{20}) - \frac{1}{3} S(L_{48})\}^2,$$

and for block III by

$$\frac{3}{2} \{S(H_{27}) + S(H_{58}) - S(G_{27}) - S(G_{58}) + \frac{1}{3} S(L_{27}) - \frac{1}{3} S(L_{58})\}^2,$$

where $S(H_{27})$ indicates the sum of the hogs of litter 27, etc. It will be realised that these are the sums of squares that are to be subtracted from the total sum of squares to give the error, but they are now no longer appropriate for testing sex effects.

In order to see how the experiment was going a preliminary analysis of block I was undertaken after it had been running for six weeks. Table III gives the initial weights and the weights after six weeks. Table IV gives the analysis of variance of the initial and experimental weights, the corresponding analysis of covariance, and the analysis of variance of the experimental weights with the effects of varying initial weight eliminated. At the bottom of Table IV is given a summary of the treatment effects, adjusted for initial weight, together with their standard errors.

Table III.

Block I initial weights (lb.) (mean weight: 35.0 lb.).

	9 H	9 G	12 H	12 G	19 H	19 G	21 H	21 G	Total
DG	21	24	37	25	—	45	40	—	192
WG	—	28	29	—	47	50	30	37	221
D	26	—	—	30	60	39	38	20	222
W	24	30	25	27	61	—	—	37	204
Total	153		173		302		211		839

2's, 289; 4's, 296; 8's, 254; H, 438; G, 401.

Block I weights after six weeks (lb.) (mean weight: 68.6 lb.).

	9 H	9 G	12 H	12 G	19 H	19 G	21 H	21 G	Total
DG	31	46	75	45	—	80	76	—	353
WG	—	58	60	—	116	103	82	93	512
D	52	—	—	55	98	65	84	39	393
W	35	54	46	54	116	—	—	83	388
Total	276		335		578		457		1646

2's, 567; 4's, 580; 8's, 499; H, 871; G, 775.

Table IV. *Analysis of variance, block I. Initial weights and experimental weights after six weeks.*

	Degrees of freedom	Sums of squares and products				Mean square
		(In.) ²	In. × Exp.	(Exp.) ²	(Exp. - <i>b</i> In.) ²	
Company	2	126.58	+ 244.70	473.08	0.92	0.46
Wet v. dry	1	5.04	+ 70.58	988.17	724.03	724.03 (- 8.51)
Green food	1	7.04	- 45.50	294.00	506.12	506.12 (- 8.27)
W × G	1	11.02	- 16.29	24.08	134.58	134.58
Litters	3	2180.46	+ 4319.58	9000.84	446.37	148.79
Sex	1	57.04	+ 148.00	384.00	19.06	19.06
<i>b</i>	1				1723.02	1723.02
Error	13	423.78	+ 854.51	2905.67	1182.65	90.97
Total	23	2810.96	+ 5575.58	14069.84		

Increase in experimental weight per lb. of initial weight (*b*) = +2.0164 lb.Interaction W × G (Q'): In. = -2.875 lb.; Exp. = 4.25 lb.; Exp. - *b* In. = +10.05 lb. ± 8.24 lb.

Increase per pig due to wet as against dry feeding (adjusted for initial weight)

= $\frac{1}{2}$ (512 + 388 - 353 - 393) - $b \frac{1}{2}$ (221 + 204 - 192 - 222) = 10.98 lb. ± 3.89 lb.

Increase per pig due to green food (adjusted for initial weight) = 9.18 lb. ± 3.89 lb.

In testing the significance of treatment effects after elimination of differences of initial weight the comparison of the treatment mean squares derived from the analysis (Exp. - *b* In.)² with the error mean square by means of the *z* test is not strictly correct, since the corresponding degrees of freedom are not orthogonal with the regression (*b*) degree of freedom. Fisher deals with this point (and the parallel disturbance to the standard errors) in (3), where he gives an elegant method

of computing the correct treatment mean square. Alternatively, the appropriate corrections to the treatment mean squares of the analysis of $(\text{Exp.} - b \text{ In.})^2$ can be obtained. If E_{xx} , T_{xx} , are the sums of squares in the analysis of the initial weights for error and the treatment group to be tested (n_t D.F.) respectively, and T_{xy} is the corresponding treatment sum of products, b being the ordinary estimate of the regression coefficient, the correction to the treatment *mean* square is

$$-\frac{(T_{xy} - bT_{xx})^2}{n_t(E_{xx} + T_{xx})}.$$

Since this correction is always negative there is no need to trouble with apparently non-significant effects. With a reasonable number of degrees of freedom for error it is usually small and can be computed on a slide rule. In the analyses of this paper the corrections are given in brackets. All are quite negligible.

There are several points of interest in the analysis under consideration. The pigs on wet food already show a significantly greater live-weight gain than those on dry food. Green food is also significantly beneficial, though the full effects of lack of green food are not yet apparent. The interaction between the feeding treatments is not significant. The difference between litters, which is very large (being chiefly an age effect), is nevertheless almost eliminated by allowing for initial weight. Variation due to both sex and numbers in pen (sub-blocks) is subnormal.

The error variance has been reduced to less than half its original value by the elimination of initial weight. The standard error per pig is 9.5 lb., which is 13.9 per cent. of the final weight or 28.4 per cent. of the increase in weight. These values are undoubtedly inflated by the imminent failure of pigs without green food, and by the fact that the obviously weakly hog of litter 9 on dry and green food was not excluded. But even without these causes a relatively high percentage standard error is of course to be expected when only a few weeks are taken, for temporary disturbances at the start of the experiment, and small irregularities in the growth rate, assume a much greater importance.

ANALYSIS AND DISCUSSION OF FINAL RESULTS.

The most striking, and disconcerting, feature of the experiment was the failure of pigs without green food. Of the thirty-six pigs without green food thirteen were removed from the experiment before its completion and fifteen of the twenty-three remaining lost weight during two or more weeks (not necessarily consecutive). Of the thirty-six pigs re-

ceiving green food only three (all on dry food) were removed from the experiment, and four more (two on dry, two on wet food) lost weight during two or more weeks. The pigs without green food would probably have done even worse but for the circumstance that pigs not thriving were run out in the fields for a day or two, so that any green-food deficiency was doubtless temporarily made up. No specific disease was detected, but clearly green food is a vital necessity to the health of pigs kept under these conditions.

The failure of the pigs which received no green food necessitated their rejection from the analysis of the final weights. This would have made the final analysis very troublesome were it not that both sex and numbers in pen proved to have an entirely negligible effect.

Table V exhibits the comparisons between pigs of opposite sex but of the same litter and feeding treatment. The final difference after correcting for initial differences by means of the regression coefficient determined later is only +1.41 lb., with a standard error (also determined later) of ± 6.04 lb. There is thus no trace of any sex difference.

Table V. *Differences due to sex. Pigs in same litter and receiving same feeding.*

Live weights (lb.): hogs (♂) minus gilts (♀).			
	Initial	Final	Final - <i>b</i> Initial
	+12	+76	+31.8
	- 3	- 3	+ 8.0
	- 7	- 7	+18.8
	+ 3	-20	-31.0
	- 8	-34	- 4.5
	- 3	- 3	+ 8.0
	+15	+34	-21.2
Mean	+ 1.3	+ 6.1	+ 1.41 ± 6.04

Variation of numbers in pen likewise showed no effects. The elimination of any such effects in the analysis is not complicated by the failure of pigs without green food, but it is made more difficult by the failure of some of the pigs receiving green food. It was therefore omitted from the first analysis and its magnitude examined with the aid of the results obtained. No differences were discovered, and no adjustment of the first analysis was made.

It should perhaps be mentioned here that ignoring factors the effects of which the experiment was designed to eliminate, merely because no such effects appear to exist, will produce estimates of error which are biased, being on the average too small. Such a procedure should be

adopted only in exceptional cases where the original design has more or less broken down.

With the neglect of sex and of numbers in pen the analysis of the pigs receiving green food becomes exceedingly simple. It may best be illustrated on the initial and final weights of block I. These are shown in Table VI.

In any litter having a single pig on wet feeding and a single pig on dry feeding the estimate of the feeding difference is merely the difference of the weights of the pigs. If there are two pigs on dry feeding the corresponding estimate is the difference of the mean of the weights of these two pigs and the weight of the pig on wet food, and similarly with two pigs on wet feeding. These differences are shown in Table VI together with their relative weights. The weighted mean of these differences gives the efficient estimate of feeding difference.

Table VI. *Initial and final weights of pigs on green food, block I.*

Litter	Initial				Final			
	9	12	19	21	9	12	19	21
Dry	24	37	45	40	151	218	191	205
		25				142		
Wet	28	29	47	30	194	190	229	205
			50	37			232	212
$W - D$	+4	-2	+3.5	-6.5	+43	+10	+39.5	+3.5
Relative weight	$\frac{1}{2}$	$\frac{2}{3}$	$\frac{2}{3}$	$\frac{2}{3}$	$\frac{1}{2}$	$\frac{2}{3}$	$\frac{2}{3}$	$\frac{2}{3}$
Weighted mean			-0.533				+22.733	

In the analysis of variance the feeding effect is represented by the square of this weighted mean multiplied by the sum of the relative weights. The error can be estimated from two sources: (a) differences between the estimates of the treatment difference from the various litters, for which the sum of squares is computed by multiplying the square of each estimate by its relative weight, summing, and subtracting the feeding effect square already calculated; (b) differences between pigs in the same litter and on the same feeding, for which the sum of squares is computed by taking half the square of each such difference and summing.

This is the whole process of analysis. The results, as far as block I is concerned, are shown in Table VII, where the analysis with effect of initial weight eliminated is also given.

The results from the three blocks can be combined into one analysis, provided that we are prepared to assume that the error variance is constant from block to block. This does in fact appear to be substantially the case, and the assumption was therefore made. No pigs were rejected,

other than those already rejected by the farm staff, except two pigs of the same litter of block III. These two pigs were both receiving wet food, and the dry-food pig had already been rejected.

The three estimates of the regression of final on initial weight from the three blocks are not significantly different. The values are shown in Table VIII. The combined estimate, 3.68, was therefore adopted in the adjustment of the final results for differences of initial weight.

Table VII. *Analysis of variance and covariance, block I.*

Sums of squares and products.

	Degrees of freedom	(Initial) ²	Initial × final	(Final) ²
Treatment	1	0.71	- 30.31	1292.01
Error (a)	3	46.29	189.98	747.49
(b)	3	101.00	475.00	2917.00
Total error	6	147.29	664.98	3664.49

Regression of final on initial weight (b) = +4.5148.

Analysis of variance of final weight adjusted for differences of initial weight.

	Degrees of freedom	Sum of squares	Mean square	
Treatment	1	1580.17	1580.17	(- 7.59)
Error	5	662.26	132.45	

Table VIII. *Summary of results.*

	Block -			Mean or total
	I	II	III	
Number of pigs rejected	1	1	3	5
Time (weeks)	21	22	20	21
Commenced	Apr. 11	May 10	June 15	—
Average age at start (weeks)	11.1	11.2	10.5	10.9
Average weight (lb.): at start	35.6	32.9	41.2	36.6
at end	197.2	178.4	180.1	185.2
Regression of final on initial weight	4.51	3.52	2.81	3.68
Standard error	±0.930	±1.59	±0.985	±0.623
Increase per pig per week (lb.):				
Mean of wet and dry	7.70	6.61	6.94	7.08
Difference (W - D)	+1.18	+1.23	+2.47	+1.63
Standard error	±0.339	±0.324	±0.398	±0.205
Food per 1 lb. increase (lb.):				
Mean of wet and dry	4.306	4.596	4.504	4.469
Difference (W - D)	+0.334	+0.196	-0.159	+0.124
Standard error	±0.234	±0.234	±0.262	±0.141

The standard errors of the final weight of a single pig after adjusting for initial weight (16 degrees of freedom) was found to be 11.3 lb., which is 7.6 per cent. of the average increase in weight.

We are now in a position to consider the effect of wet *v.* dry feeding.

Wet feeding produces significantly greater increases of weight than dry feeding. The mean increases per pig per week for the three blocks, after adjustment for initial weight, are given in Table VIII. The pigs grew at decidedly different rates in the three blocks, and the difference between wet and dry feeding is significantly greater in the third block than in the other two.

This difference between wet and dry feeding is easily explicable. The pigs found the wet food more appetising, and consumed more of it. The mean food consumption per 1 lb. live-weight increase for the three blocks (given in Table VIII) is greater for the wet feeding in the first two blocks, but there is a reversal of the difference in the third block and none of the differences approaches significance. The efficiency of food utilisation, therefore, was substantially the same for wet and dry feeding.

The effect of dry feeding, consequently, is likely to be similar to that of rationing.¹ If there is no more efficient utilisation of the food with rationed feeding then clearly *ad lib.* feeding will be the most profitable, for it is both less trouble and enables a quicker turnover of pigs to be made, with a consequent reduction in overhead expenses, labour charges, etc.

General considerations would indicate that the faster the pig grows the smaller will be the proportion of the digested food required for maintenance. Consequently unless a considerable fraction of the food is undigested in *ad lib.* feeding it would appear that this system of feeding is likely to be the most efficient. The preliminary results of a further experiment now in progress, which is designed to investigate the effects of rationed feeding, confirm this argument, showing the highest efficiency with *ad lib.* feeding.

The above analysis depends on the assumption that numbers in pen have no appreciable effect. We can now demonstrate that this is the case. Table IX shows the final weights of block I set out by litters and numbers in pen. In litter 9 the pig with eight in pen is missing. The weights of the other pigs with eight in pen have therefore been adjusted by one-half the estimated difference per pig between wet and dry feeding for this block, or 11.37, this being added to the weights of those receiving dry food, and subtracted from those receiving wet food. The formula for estimating the yield of a missing plot (1) may now be used to supply a weight for the missing pig; this is

$$x = \frac{345 \times 4 + 568 \times 3 - 2158}{(4-1)(3-1)} = 154.$$

¹ See footnote on p. 515.

Substituting this value we obtain the totals 810, 780 and 722 as the total final weights, equalised for litter and feeding, of pigs running two, four, and eight in a pen respectively. Performing the same sequence of operations on the initial weights, and then using the corresponding resultant totals of the initial weights to correct the final weights by means of the regression of final on initial weights, afterwards dividing by four, we obtain estimates of pen effects with the effects of differences of initial weight eliminated.

Table IX. *Computation of effects of numbers in pen.*
Block I. Final weights.

	Litter				Total
	9	12	19	21	
Two in pen	151 (D)	218 (D)	229 (W)	212 (W)	810
Four in pen	194 (W)	190 (W)	191 (D)	205 (D)	780
Eight in pen	<i>x</i>	153* (D)	221* (W)	194* (W)	568
Total	345	561	641	611	2158

* Adjusted for type of feeding (wet or dry).

Table X gives the result of this calculation for the three blocks. The experimental standard error of the mean of four pigs, ± 5.7 lb., is amply large enough to account for the differences observed; clearly therefore neither numbers in pen nor any other factors associated with these sub-blocks produced any appreciable effects.

Table X. *Effects of numbers in pen. Final weights adjusted for differences of initial weight.*

	Block			Mean
	I	II	III	
Two in pen	196.1	173.4	185.7	185.1
Four in pen	191.3	179.0	175.4	181.9
Eight in pen	190.6	179.9	179.2	183.2

In this discussion of the results we have only taken account of the initial and final weights, and total food consumption. There is, however, a large mass of further data available, namely, the intermediate weekly weighings and weekly food consumption. The question remains as to whether there is any additional information to be obtained from this set of data.

That there is little such information is indicated by inspection of the growth curves of the individual pigs. Fig. 1 shows those of litter 21. The growth rate of the pigs receiving green food is remarkably constant,

and there is no indication of any differences between the two treatments other than those revealed by the final weights.

A further interesting point revealed by the growth curves is that any temporary disturbance to regular growth tends to correct itself subsequently. Thus a pig that has suffered a check tends to grow more rapidly for a few weeks till it has made up lost ground.

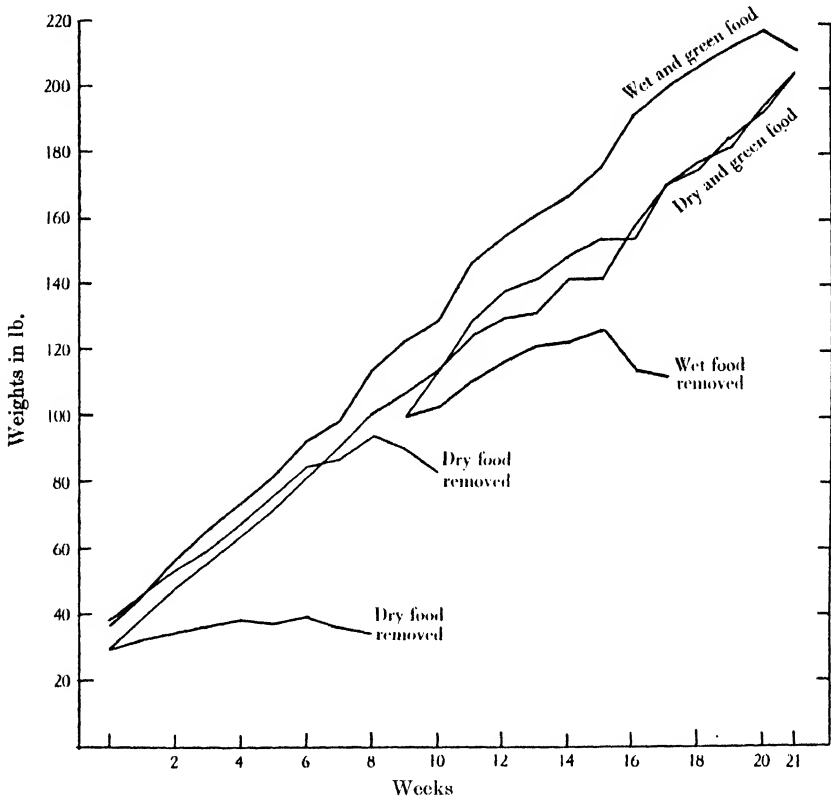


Fig. 1. Growth of pigs in litter 21. The first nine weeks of the pig on wet food and of one of the pigs on dry and green food have been omitted to clarify the diagram.

The intermediate weights are useful as an index of the progress of each pig and they enable definitely defective pigs to be eliminated, but it is doubtful whether any correction for temporary checks, at any rate in *ad lib.* feeding, is justified, especially in view of the apparent later compensation.

All our analyses have been based on the first and last weighings only. A single weighing, however, may be affected by temporary irregularities which will average out when a few consecutive weighings are taken.

It is possible, therefore, that more precise results might be obtained by taking the mean of two or more weighings at the beginning and end of the experiment. For such a procedure to be valid all the pigs would require to be kept for a preliminary period without treatment. This was not done in this experiment, but we may nevertheless determine the error from such meaned results and compare it with that already obtained. This error, taking the means of the two initial and the means of the two final weights, as shown, was found to be 9.9 lb., as compared with 11.3 lb. There thus does appear to be a real, if only small, reduction in error.

The various standard errors obtained in this experiment are summarised in Table XI. Remembering that the amount of information is inversely proportional to the square of the standard error, we see that elimination of initial weight has more than doubled the information from the experiment, and that taking the mean of two initial and two final weights has increased the information by a further 20 per cent. over that obtained with initial weight eliminated.

Table XI. *Standard errors of total live-weight increase*
(per pig, in lb. and per cent. of increase).

Without elimination of differences of initial weight	16.1 lb. or 10.8 %
With elimination of differences of initial weight	11.3 lb. or 7.60 %
With elimination of initial weight and food consumption	9.3 lb. or 6.29 %
Means of two initial and two final weights (initial weight eliminated)	9.9 lb. or 7.02 %

COMPARISON WITH DUNLOP'S RESULTS.

Dunlop, in his Cambridge experiments, used a radically different method of experimentation. Individual feeding was used, but instead of *ad lib.* feeding each pig was rationed, the ration of each week depending on the weight of the pig at the beginning of the week. The food allowances were on such a scale that the worst feeders would consume the whole of their ration. Each pig was started on the experimental ration when its weight reached a given value and was then fed on this ration for a given number of weeks.

Using this technique Dunlop obtained remarkably accurate results. He gives the standard error per pig (running for twelve weeks after reaching a weight of 64 lb.) calculated from three experiments (70 pigs, 55 degrees of freedom) as 3.35 lb. This is 3.68 per cent. of the average increase of weight, 91.1 lb. Comparable figures calculated from the Rothamsted experiment give a standard error of 10.5 lb. on an average increase of 88.5 lb. above 64 lb., *i.e.* 11.9 per cent.

The standard errors given by Dunlop are, moreover, overestimates, as he himself admits, for litter effects have not been eliminated, although litter was equalised as far as possible over the different treatments. His errors are computed merely from the deviations from treatment means, ignoring litter and other factors altogether. These errors are consequently considerably biased, since the original design of the experiments consisted of 5×5 Graeco-Latin squares involving five litters of five pigs each, in five pens, there being also equalisation for position of feeding pen. The actual design instanced was:

	Pen P					Pen Q					Pen R				
Position in pen	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Litter	4	2	5	1	3	2	5	3	4	1	5	3	1	2	4
Treatment	A	D	B	C	E	B	E	C	D	A	C	A	D	E	B

	Pen S					Pen T				
Position in pen	1	2	3	4	5	1	2	3	4	5
Litter	3	1	4	5	2	1	4	2	3	5
Treatment	D	B	E	A	C	E	C	A	B	D

In such a design a valid estimate of error (8 degrees of freedom) would be obtained on eliminating litters, treatments, pens, and position of feeding pen (4 degrees of freedom each), since these sets of degrees of freedom are all mutually orthogonal. Unfortunately, the litters available did not all contain five suitable pigs, and deficiencies were supplemented by pigs from other litters, so that a partial equalisation of litters was attained, with consequent non-orthogonality of litters, treatments and the other factors. This makes the determination of any error difficult and introduces a very troublesome type of disturbance into the treatment means. The difficulty could only have been avoided by complete redesign, the simplest procedure (though perhaps not the most efficient) being to ignore litters altogether, assigning pigs to their pens and treatments at random.

The trouble has arisen owing to neglect of what we gave as the first requirement of experimental design, namely, that the experiment should be capable of furnishing a valid and unbiased estimate of error. Ignoring pens, about which no particulars are given, it is possible to obtain unbiased estimates of error by direct analysis of the second experiment, and by the rejection of one value in the third experiment and the inclusion of another by means of the missing-plot technique. In the first experiment the fitting of constants must be resorted to. Table XII shows that the unbiased estimates of error are considerably less than those given by Dunlop. In the first and third experiments the litter effects

are definitely significant, and in the second experiment are almost significant.

The true values of Dunlop's errors per pig are thus even lower than he claims, and far lower than the errors of the Rothamsted experiment. Dunlop attributes these remarkably low errors to the system of rationing adopted. On the face of it this is possible, for clearly the heavier feeders will grow more quickly, and such differences in growth rate will increase the experimental error. But if differences in food consumption are the main cause of the higher variability in the Rothamsted experiment a considerable reduction should be effected by adjusting the final weights for differences in food consumption by the analysis of covariance. This adjustment was performed on the weights twelve weeks after reaching 64 lb., but the reduction in error, from 10.5 to 9.1 lb. per pig, was quite small. The corresponding figures from Table XI are 11.3 and 9.3 lb. We must therefore postulate that the main cause of the high variability was variation in efficiency of food utilisation. It seems unlikely that had the Rothamsted pigs been rationed almost the whole of this variability would have disappeared.

Table XII. *Errors in Dunlop's experiments.*

Experiment	Duration weeks	Initial weight lb.	Mean increase lb.	Errors per pig		
				Dunlop's estimate lb.	Corrected estimate lb.	Corrected estimate % of increase
Spring 1932	12	64	92.5	4.67*	2.81	3.04
Autumn 1932	17	40	128.0	5.00	4.25	3.32
Spring 1933	12	45	85.4	2.54	1.70	1.99

* Apparently computed wrongly. Should be 4.40.

We are thus forced to the conclusion, on the evidence before us, that part at any rate of the difference in variability must be attributed to the greater homogeneity of material and the better experimental conditions at Cambridge. Whether any of the factors making for higher error at Rothamsted are controllable it is at the moment impossible to say, but it is eloquent testimony to the extreme care with which the Cambridge experiment must have been executed that such low errors were possible by any method¹.

Further information will shortly be available on the reduction of

¹ See also (1). The series of experiments there described, carried out at McGill University, are comparable with the Rothamsted experiment, except that the effects of litters were not eliminated. Rather higher errors were obtained than at Rothamsted.

error likely to be effected by rationing from the rationing experiment now in progress at Rothamsted. In the meantime the greatest caution should be exercised in using the two sets of results as a test of the relative merits of the two methods from the point of view of accuracy.

Even if the rationed system of experimentation should prove very much the more accurate (which, as we have said, appears unlikely), there remains the vital objection that the experimental conditions are highly artificial. Every departure from ordinary farming conditions makes the experimental conclusions reached of less practical interest and validity, and in many experiments on questions of economic management the whole point of the experiment would be missed with rationed feeding. The difference between wet and dry feeding, for example, would have been completely obscured had the rationed system been adopted.

SUGGESTED IMPROVEMENTS IN EXPERIMENTAL TECHNIQUE.

It is probable that the Rothamsted experiment was too complicated. Leaving aside the failure of the pigs without green food, which was not due to any fault of the experiment, it is clear that the balance of design of this type can be very seriously upset by the failure of a small percentage of the pigs. The interpretation of the results of the present experiment would have been rather troublesome were it not for the fact that neither sex nor numbers in pen had any appreciable effect.

In order to mitigate the disturbance due to the failure of individual pigs, spare pigs on neutral treatment may be carried. This was actually done in the present experiment, but sufficient attention was not given to their selection, and they were none of them from the same litters as the experimental pigs. In view of the significance of litter differences, therefore, results obtained from them were of no value. This has been remedied in the second experiment by carrying one spare pig of each litter for substitution should any pig of that litter fail.

Spare pigs, however, are apt to provide unsatisfactory data. They receive the experimental treatment for only a part of the time, and a pig that is eventually written off as a failure often fails gradually and must be considered defective from the start. The design, therefore, should be kept as simple as possible, in order to enable missing data to be compensated for by the missing-plot technique. Considerable simplification of the Rothamsted design could have been made if sex had been ignored and investigations of the effects of variation of numbers in pen omitted. Litters of four might then have been used, the whole of each litter being run in one pen (one pig of each litter receiving each treatment). Litter

and pen effects would then be confounded with consequent simplification of the analysis. Similarly in a 3×2 experiment litters of six pigs could be utilised, and in a $2 \times 2 \times 2$ experiment litters and pens of four with the second-order interaction confounded.

Another possibility which has not so far been considered is to use a group of pigs instead of a single pig as an experimental unit. With groups of four, for example, each unit could be run in a separate pen, with the pens arranged in randomised blocks. The experiment would have to be a large one in order to provide a sufficient number of degrees of freedom for experimental error, for the analysis must be based on the totals of each unit and not on the individual pigs. On the other hand, in view of the small pen effects actually observed, the efficiency of the experiment measured on the basis of a single pig will probably be about the same as with individual feeding, at least with *ad lib.* feeding where competition at the feeding trough does not come into play. The great advantage of this method of experimentation is that the labour of feeding is very considerably reduced, and less elaborate pens are required. Where plenty of material is available it may be the most efficient method. The radical difference between this and the old group-feeding method is that in the old method there was entirely inadequate or no replication of the groups. With adequate replication and proper randomisation the conclusions can be made as statistically valid as those reached by individual feeding.

SUMMARY.

1. The conditions which must be fulfilled in the design of an animal husbandry experiment if the results are to be statistically valid are summarised, together with possible methods of improving the efficiency by suitable design.

2. The results of a pig-feeding experiment conducted at Rothamsted are discussed. The experiment showed that green food was essential to young pigs under the conditions of the experiment, that pigs fed on a wet mash grew at a faster rate than pigs fed on dry meal, the difference being due to the greater quantity of food consumed by the former, and that the effects of numbers in a pen (giving equal floor space per pig) were negligible in spite of the greater possibilities of exercise in the larger pens.

3. The various standard errors of the experiment are evaluated and compared with the results of Dunlop's experiments at Cambridge. It is pointed out that the considerably lower errors of the Cambridge experi-

ment, attributed by Dunlop to his method of rationed feeding, may be due to other causes.

4. Possible modifications of the present type of design are discussed.

In conclusion I should like to mention that this paper was originally planned as a joint one with the late Mr H. G. Miller, the Farm Director at Rothamsted, who was in charge throughout of the practical execution of the experiment. His illness and untimely death, however, prevented him from taking any part in it.

REFERENCES.

- (1) CRAMPTON, E. W. and HOPKINS, J. W. The use of the method of partial regression in the analysis of comparative feeding trial data. Part I. *J. Nutrition* (1934), **8**, 113.
- (2) DUNLOP, G. Methods of experimentation in animal nutrition. *J. Agric. Sci.* (1933), **23**, 580.
- (3) FISHER, R. A. *Statistical Methods for Research Workers*. 5th edition. (In press.)
- (4) YATES, F. The analysis of replicated experiments when the field results are incomplete. *Emp. J. Exp. Agric.* (1933), **1**, 129.

(Received May 23rd, 1934.)

SOME INDUCTIVE METHODS IN PEDOLOGY

E. M. CROWTHER

*(Chemistry Department, Rothamsted Experimental Station,
Harpenden, Herts)*

Descriptions of soils and analytical data are necessarily based on current theories of soil genetics and soil fertility, even though the underlying theories are rarely stated explicitly. In field work and detailed mapping, the surveyor builds up his concept of each soil type from the characters of isolated profiles, but in attempts to develop more comprehensive systems of classification or to relate soils of different countries deductive rather than inductive methods are generally employed. There appears to be a definite danger that important observations and relationships may be missed through the uncritical use of too restricted theories of soil genetics. Thus, in some general systems of soil classification, undue weight is given to isolated factors, such as climate, exchangeable bases, or clay composition. Confusion has already arisen from attempts to define "solonetz" soils in terms of exchangeable sodium, and "laterites" by the $\text{SiO}_2:\text{R}_2\text{O}_3$ ratios of their clay fractions. Obviously, chemical compositions and environmental factors must be considered in any interpretation of pedogenic processes, but it is doubtful whether any system of classification based on the dominance of a single factor or property is yet sufficiently well-established for general application. The present paper contains some proposals for supplementary methods of collecting and analysing soil

data with the object of placing soil classification on a firmer inductive basis.

SOIL NOMENCLATURE

The Society will consider at Oxford a proposal to set up for each Commission an International Nomenclature Committee to define and translate technical terms. For the Fifth Commission there would appear to be especial value in compiling definitions and detailed descriptions of important kinds of soil which have been recognised and named in common speech in many countries. If a characteristic soil type is sufficiently widespread to have acquired a common local name, it seems certain that a genetic classification must ultimately find room for this type as a natural unit. It is sometimes forgotten that the accepted major world groups of soils were named in this way in bygone ages. Such words as "podsol," "chernozem," "solontschak" lose a little of their academic glamour when it is realised that they are merely Russian peasant words for "ash underneath," "black soil," "much salt," respectively. These well-established local names have, however, proved their value in many other parts of the world, whilst some more recent inventions, such as "brown earth," and "laterite," with neither tradition nor rigid definition behind them, appear to have caused as much confusion as clarification. A collection of definitions of local soil names would serve as a useful dictionary in reading foreign literature, and, in addition, would reveal many natural units which must be accommodated in any general soil classification. If it should happen that the terms of one region emphasised a single pedogenic factor, then there would be strong presumptive evidence of the dominant importance of that factor in the region and a warning of the danger of endeavouring to use a general scheme based on the dominant factors of some other region.

In Great Britain, the traditional names for soils emphasise either the nature of the parent material, *e.g.* "stonebrash," "brick-earth," "clay-with-flints," or the relatively recent geological history, *e.g.* "carr," "carse," "fen." Some of the county soil maps of over a century ago could serve as outline geological maps, and many of the clays and sands so mapped have since been named by the geologists in a scheme of nomenclature resembling that of the American soil survey, *e.g.* "London Clay," "Oxford Clay." There can be no doubt, therefore, of the importance of geological factors in the classification of British soils. On the other hand, there is a conspicuous absence of names for soils now recognised as belonging to the major world groups. Thus, the fact that there is no English equivalent for "podsol," except perhaps "heath soil" or the obsolete words "fox earth" or "fox bench," shows that this soil type is not important among our cultivated soils. It may prove that the "hazel mould" of the old English writers was a more picturesque anticipation of "brown earth," but the term has gone out of use. "Downland soil" or "limestone soil" have fairly clear meanings in England, but the collaborators in the soil map of Europe were left uncertain whether these soils should be mapped as "rendzina," for this term was not clearly defined in the theoretical classification adopted for the map. Would it not be wise to collect descriptions of soils which are already

well-recognised and named before attempting to lay down comprehensive systems of nomenclature and mapping?

A few other names already used in English soil literature may be listed without comment to illustrate the need and value of definition: "adobe," "gumbo," "prairie" from America; "vlei," "badob," "ilepa," "murram" from Africa; "mallee" from Australia; "regur," "black cotton soil," "paddy" from India. Such lists could easily be expanded to provide material which would serve as a valuable supplement to the rapidly developing vocabulary of names of places for type specimens and of pedogenic processes for more academic descriptions.

TIME SCALES

Empirical mapping for land valuation or advisory work and detailed studies of individual soils often fail to provide material which can be correlated with observations in other regions. It is suggested that it might be found useful to attempt to express the genetic interpretations of typical soils in terms of a logarithmic time scale analogous to the pH scale (*i.e.*, negative logarithms to the base 10 of time in years). In Great Britain many cultivated soils would reveal the effects of factors spread fairly regularly along this scale. Thus, recent cultivations, including such drastic treatments as deep ploughing or "gyrotilling" might occur up to 1, draining systems, periodic chalking, and alternations of grass and arable cultivation might come between 1 and 3, and the earliest clearing of the forest between 3 and 4. The climate might without gross error be taken as similar to the recorded one up to about 3.5. The present topography was largely determined between 5 and 7, and, if necessary, numbers up to about 10 could serve on a conventional scale for the age of the underlying rock.

The soil retains impressions from factors operating at all periods of its history, and the genetic theory of soil classification requires that one should endeavour to determine that history as fully as possible. In any single region complexes of soils may be found with most of their historical factors in common. Within such complexes the more empirical systems of classification and mapping for advisory purposes emphasise the differences between associated soils, but they tend to obscure the common factors which become important when the soils are to be linked up with those of other regions. The use of such a time scale might serve the important philosophical purpose of directing attention to the abstractions employed in treating the soils of natural regions. It would remove that vague fiction "the virgin state." It would show the arbitrariness of accepting the rodent as a factor in the formation of chernozem whilst neglecting the effects of man and his domesticated animals, and thus lead one to anticipate that countries with early domestication of animals and early agriculture (*e.g.*, Southern Russia) would have different soils from regions (*e.g.*, North America) with comparable climates but much later serious disturbance by man. Observations on laterite and podsol profiles are made differently by those who think only of current processes and by those, such as Prescott in Australia and Beijerinck in Holland, who believe that some soil horizons are of widely different geological

age from others associated with them. The pT scale would stimulate geomorphological and geochemical work and check the premature use of completed soil systems. It is true that many experienced pedologists have no need for conventional scales in which to express their normal methods of working, but it must be remembered that many non-specialists, particularly in partially surveyed countries, have unique opportunities for obtaining valuable observations which might be missed if they continue to work from crude statements of restricted genetic theories.

SOIL ZONES AND CLIMATE

Where the soil types have been recognised and mapped over a wide region, it is possible to study inductively the dominance of soil-forming factors, and to test whether or not the distribution of soils is, in fact, in harmony with that of some independent external factor. Soil maps in Great Britain would reveal some regions in which the soils closely followed the solid geology, and others, especially those with miscellaneous drifts, in which the geological factors, as at present mapped, would be quite inadequate to account for the distribution of soils. In European U.S.S.R. it is claimed that many of the soils are in substantial equilibrium with climatic factors, and this claim can readily be tested objectively. Since the seasonal cycles of rainfall (R) and temperature (T) are similar throughout this area, the annual means will characterise the climate with sufficient accuracy to test the correlation of soil and climate. The author has published elsewhere (1), and will exhibit at Oxford, diagrams showing the mean annual rainfall plotted against the mean annual temperature for some 300 points read off at equal distances over 20° of latitude and 30° of longitude, and grouped according to 12 soil types in Prassolov's soil map on the scale of 1:2,500,000. If the soil types were in equilibrium with climate, then there would be a characteristic band of rainfall or temperature, or alternatively some band of regression of rainfall on temperature for each soil type. In the large area covered by podsolised soils (including bog, peaty and gleyed soils) it was found that the rainfall increased significantly at the rate of 1.6 cms. per degree C. increase in temperature. Similarly, for the ordinary chernozem and the southern chernozem belts, the rainfall increased significantly at the rate of 1.2 cms. per $^\circ\text{C}$. Between these two belts there were irregularly distributed belts of degraded grey and brown soils, leached chernozems, and deep chernozems. One may conclude then from the distribution alone, that these transitional soils are either intrazonal or local. Presumably their occurrence depends on other historical or geographical factors involving the date of the retreat of the forest from the steppe. The chestnut soils cover about the same temperature range as the other soils, but have a much narrower rainfall range, i.e., their distribution depends primarily on rainfall. Empirically it was observed that the boundaries of most of the soil types could be expressed by convenient round numbers for the ratio $(R-30)$ cms. to $(T+4) ^\circ\text{C}$. This ratio was negative for brown semi-desert and alkali soils, and increased steadily to over 3 for the podsolised soils.

The Azov chernozem is quite abnormal in its distribution, covering a wide rainfall range in a narrow band of high temperature. It clearly

depends on some local geographical condition not sufficiently expressed by climate. This is, in fact, clear from its name, for the Russians would not follow the American system of place names unless they were compelled by some local condition to do so.

A purely inductive treatment of distribution thus brings out the major characteristics of the Russian soil zones, and shows which ones are sufficiently widely and regularly spread to be regarded as in substantial equilibrium with climatic factors, and which are not. The inductive method has great advantages over the more usual deductive treatment—as *e.g.*, Lang's *Regenfaktor* and Meyer's *N.S.Q.*—for it affords a direct test of any conclusions drawn. By plotting the rainfall against temperature for sample points within a soil zone, one not merely sees that the complex climatic factors deduced *a priori* fail to account for the actual distributions, but one also realises the importance of the absolute values. The mean annual rainfalls and temperatures of the parts of U.S.S.R. and U.S.A. which have already been surveyed, rarely overlap, and although one might expect certain similarities in soil types, it would be contrary to genetic theory to expect identities.

REFERENCE

- ¹ Ministry of Agriculture Conference on Agricultural Meteorology, 1931.

THE FUNCTIONS OF MECHANICAL POWER IN SOIL CULTIVATION.

By B. A. KEEN, D.Sc.*

(December, 1934.)

NOTE :—*See Appendix, pages 190, 191, for a glossary of Agricultural Terms used in the paper.*

INTRODUCTION.

THIS paper is intended to supply what may be called the agricultural background for Mr. S. J. Wright's technical discussion of the problems connected with the use of internal combustion engines in agriculture. Both papers are specifically confined to one aspect of farm activity—the use of power in soil cultivation. There is full justification for this limitation : to the arable farmer, soil cultivation is the most important section of his work ; he must cultivate, and cultivate as efficiently as the variable weather conditions will permit, before he can even begin the later series of operations that ultimately give him his saleable produce.

From time immemorial the farmer has used animals for farm work. All his methods and the implements devised for them were developed for haulage by oxen and later by horses. His use of any kind of mechanical power for the purpose is a modern innovation, and his employment of the internal combustion engine is very modern indeed. For our purpose it must be considered in two aspects : firstly, as a power unit on wheels hauling existing types of cultivation implements through the soil—the tractor ; and, secondly, as a power unit, actuating rotating tines that break down the soil to a finer condition in one operation—the rotary cultivator. Both aspects are considered in the present paper.

The farm tractor is designed primarily to pull cultivation implements ; the belt-drive pulley and the power take-off are regarded as desirable additions which extend its range of work to driving barn machinery, harvesters, mowers, etc., without detriment to its primary function. Yet, on the average farm, the power used in cultivations is only a fraction of the yearly power consumption. It is somewhat of a paradox that tractor design should have to be primarily founded on its suitability for an operation which, in spite of its vital importance, might fairly be described as an occasional job only.

* Assistant Director, Rothamsted Experimental Station, Harpenden, Herts.

The rotary cultivator is, in its present form, a tool for even a more specific purpose. The engine and the soil miller constitute one unit : the rotary action of the engine is used to apply a rotary action to the soil. Although the present tentative experiments on attaching (either behind or in place of the miller) various other implements, such as ridging ploughs and light rollers, may ultimately lead to a satisfactory range of implements, the first problem to be solved is whether a soil tilth produced by a rotary method is equal to or better than that produced by the traditional sequence of plough, cultivator, and harrow.

To summarize the present position, it seems unlikely that tractor design will undergo revolutionary changes in the next ten to fifteen years. The farmer has had sufficient experience of it to be able to state what, ideally, he would like his tractor to do ; the essentials of these requirements are set out in the present paper with the idea that the engineer will say how far they can be met. With regard to rotary cultivation, this is, in its present stage, more a problem of soil physics than of agricultural engineering. The relative properties and field behaviour of the tilths produced by rotary cultivation and by traditional methods must first be elucidated, since this is the information on which the engineers will work. Experiments of this kind have been carried out at Rothamsted for some years and will be continued. The results so far obtained are discussed in the present paper.

HISTORICAL.

The different kinds of implements used in soil cultivation have all developed from a pointed stick whose function was to stir and break up the soil. Cultivators and harrows are in the direct line of descent from the pointed stick ; the plough represents a divergence from the line, in that its purpose is to invert the soil rather than to stir it. The extremes of plough design are the sod- or grassland-plough, which turns over an almost unbroken ribbon of soil, and the digger-breasted plough, common in Continental areas, which turns over a rough broken furrow with the maximum of disruption and mixing. Before the advent of the tractor, the design of cultivation implements and their methods of use had evolved subject to two basic considerations : a supply of cheap and abundant labour, and a forward speed of $2-2\frac{1}{2}$ m.p.h. which suited the natural walk of both horse and man.

At first the tractor had little effect on these considerations—it was regarded as a more powerful haulage agent than horses, and therefore suitable for heavy jobs, such as stubble breaking and deep ploughing. With further experience, and with the better designs of the tools for the lighter forms of cultivation, the scope of the tractor rapidly increased. The addition of such improvements as the power take-off and the development of power-operated implements for the hay crop opened up additional uses for the tractor as a farm tool. There is little doubt that a steady increase has taken place in the number of hours' work per year put in by the tractor on the average farm. Periodical censuses carried out by the Agricultural Economics

Research Institute, Oxford,* on farms employing both tractors and horses, show that the hours of work of the tractor per year on all jobs are about half those put in by the horse. There is undoubtedly room for this figure to be appreciably increased; the general introduction of rubber tyres may help here. But, desirable in many ways though this increase may be, it must be remembered that the outstanding advantage of the tractor is its ability to deal quickly with urgent work. Farming cannot be done to a rigid time-table; the weather is the controlling factor. In unfavourable seasons the farmer may be unable to work his soil when he wishes. He must produce a suitable tilth before he sows, and for this he may be compelled to wait so long that his crop, when sown at last, is almost certain to suffer in yield. It is in such conditions, and in the preparation of land for the next crop, immediately after the current one is harvested, that the tractor finds a most useful avenue of employment. Similarly, in preparing the soil for spring sown crops, the inevitable rush of work in the few fine spells in a wet spring can be tackled with some hope of success. It is not necessary to expand further this aspect; it has already been dealt with in an earlier paper.† The economic value of this reserve of power, especially to the farmer on heavy land, is incontrovertible. The tractor enables him to cut costs directly, as will be shown below, but even more important is the indirect cost cutting, through the ability to get work completed in unfavourable spells. No costings system can show the money value of indirect savings, for obvious reasons, but no farmers would dispute their importance.

FUNCTION OF POWER IN CULTIVATION.

Agricultural economists have made numerous comparisons of tractor and horse costings on the farm. In common with all agricultural costing data, they present difficulties which do not arise in other industries. Take as a simple example the cost of keeping a horse. It will be fed, wholly or partly, on food grown on the farm. What figure should be assigned to this food? It should be less than the market price of the foodstuff, but to what extent? It is not even possible to state the exact cost incurred by the farmer in growing this food, since the yield is controlled, to a degree not precisely known, by the residual value of the manures applied to the preceding crops. Some conventions must therefore be adopted, on which agricultural economists have not yet arrived at complete agreement. But, in spite of these inherent difficulties, direct comparisons of horse and tractor costings are capable of showing in what way the tractor can achieve a direct saving as compared with horse-power. Some typical results are given in the Appendix Table I, which has been constructed from figures supplied by agricultural economists. The figures, which are some years old, apply to individual farms employing both horses and tractors, and this

* See issues of "Occasional Notes" and "The Farm Economist" for the past few years.

† B. A. Keen. Proc. I.A.E., Vol. XXI, 1927, pp. 242-274.

partly accounts for the wide variation in costs for the same work. For our present purpose, however, this does not matter.

The salient feature of the table is that on all these farms tractor ploughing is cheaper than horse ploughing, while in all the other operations the reverse is the case. The explanation is simply that in ploughing the tractor is given a full load, while in the other operations it is working below its capacity. The practical implication is, therefore, that all tractor cultivation tools should be designed to give a full load like the plough. The modern tractor cultivator already does this, but there is still scope for the farmer to use gangs of harrows to increase the resistance for this naturally light type of cultivation.

The above results have an important bearing on the question of complete mechanisation of arable farming. Here it should presumably be easier to design the equipment and to operate it so that a full load is always given, although in most parts of the country extensive and perhaps costly alterations in the field boundaries would be needed.

At this stage it is convenient to point out some features of the farming industry that are not generally appreciated by those whose knowledge is confined mainly to other industries. The first is that the land itself, and not the mechanical equipment on it, is the farmer's primary machine. If he is already farming reasonably well his output per acre cannot be appreciably increased by mechanisation—he can only increase his output per man. His opportunities for expansion are therefore more limited than those of the manufacturer, who, subject only to being able to find a market for his output, can show an increased return on his increased capital. With his output at, or very near, its maximum, the farmer must look to mechanisation mainly as a means of reducing production costs, *i.e.*, of increasing his output per man. The wages of agricultural workers are low, and in view of world prices the capital a farm can carry is limited. These factors are sufficient to close to all except the largest farms the obvious line of development towards bigger and bigger machines as a means of increasing the output per man.

A second point is the impossibility, in farming operations, of fully utilising the modern practice of supplying each piece of machinery with its own prime mover of the correct horse-power. This tendency is most noticeable in the increasing use of electric motors built in as an integral part of the driven machine. But there are so many operations in the fields and buildings of the average farm that to provide the necessary range of prime movers would involve too heavy capital expenditure. In addition, there are peaks of activity and relatively idle periods whose durations cannot be predicted. Hence the tractor will often be relatively idle, and the farmer will use it for light belt work with barn machinery; from his viewpoint it is not inefficient to do so. He has his tractor, it would otherwise be idle, and he is wasting, in effect, only the difference in running cost between the tractor and a smaller engine, and he is saving the capital expenditure on the latter.

Up to this point stress has been laid on the ability of the tractor

to deal with heavy jobs, and with peak periods. Both of these considerations raise directly the question of the most suitable size and speed for farm tractors. What is the best compromise between large units working at low speeds and small units at higher speeds? To some extent this is an engineering problem, but it is appropriate here to put forward the farmer's requirements which the engineer should meet as far as is practicable.

In the first place, there is scope for a large tractor unit both on the large farm and, in the form of steam or Diesel cable cultivation sets, for contract ploughing work on smaller farms. But both the large farm and the more typical smaller holdings need a smaller unit—the general purpose tractor of about not more than 15 draw-bar h.p. So long as our farm holdings retain their present size distribution, the number of general-purpose tractors used in the country will greatly exceed that of the larger unit, and the smaller tractor is therefore the more important from the agricultural engineer's viewpoint. What characteristics should it have? To take a familiar parallel from motoring for illustration, should the tractor correspond to the heavy American car, or the baby Austin? The severe strains and the rough treatment which tractors endure require a robust construction to withstand them, but, subject to this restriction, there is no doubt that the farmer needs the smallest size of unit and the highest speed that can be given him. As already mentioned, his cultivation implements were designed for work at a walking pace, and although with more attention to design this speed has been increased, it is still on the low side. An objection often urged against increased speed is that the work required to overcome the resistance the soil offers to the cultivating tool will also rapidly increase, but actual tests do not bear this out. Soil resistance increases only slowly with speed of implement travel. Thus, in ploughing, an increase from $2\frac{1}{2}$ to 4 ft. per sec. causes only a 7 per cent increase in the draw-bar pull needed by the plough.* Hence in arriving at a compromise between the numerous conflicting requirements of tractor design the engineer has not to make provision for greatly increased resistance of the implement at higher speeds. Further objections to increased speed, often urged by farmers and some agricultural engineers,† is that the implements do bad work and are subject to excessive wear. Wear may possibly be reduced by using harder steel; but with regard to the kind of work it is obvious that a plough designed to turn a neat furrow at $2\frac{1}{2}$ m.p.h. will not give the same response at double or treble the speed. The soil will be thrown to one side and not laid in a neat furrow against the preceding one. If a smooth unbroken furrow is still required, then the curvature of the mouldboard would have to be much more gradual and its length would be greatly increased. This is obviously undesirable and impracticable. A more profitable alternative is to ascertain whether the neat unbroken and crested nature of the furrows associated with slow ploughing speeds

* B. A. Keen. Proc., I.A.E., Vol. XXI, 1927, pp. 242-274.

† Some, but not all. See N. Geldard. Implement and Machinery Review, Vol. LIV, 1928, pp. 539-540.

is an essential feature of the cultivation, or whether it is an unimportant accompaniment having merely an æsthetic value, or serving at the most as a superficial indication that the plough is set correctly to perform its real work—inversion of the furrow slice. Judged by this standard, the smooth furrow possesses little or no advantage; so long as the constituent lumps are still large and the land is left well ridged the work is good.

There is one reservation to this statement. Practically every rotation of crops includes a "seeds" ley, which ultimately has to be ploughed in. In order that the grass shall rot, the sward must be inverted to bury the vegetation completely. For this a smooth, unbroken furrow slice is essential, which, in turn, implies a gently curving mouldboard. Even if cultivating operations in general were speeded up appreciably beyond the present level, it would seem that ley ploughing would have to remain an exception, since a greatly increased length of mouldboard would be needed for this operation.

Finally, something must be said about the much discussed question whether tractors unduly compress the soil. In the early days of the tractor instances were not uncommon, for the machines were heavy and unwieldy, and the design of the land wheels was still in its infancy. The deleterious effect on the soil was, therefore, usually ascribed to excess pressure on the soil surface, although it was very probably due more to a plastic deformation of the soil under the relative motion of the strakes or spuds. It must be remembered, too, that the range of soil moisture conditions in which the tractor could be used had to be found by experience; it was not necessarily the same range as that within which cultivation by horse implements was possible. Although less is heard to-day about deleterious soil compression by tractors—in fact, numerous cases are known in which the crop grew better along the wheel tracks than elsewhere—the agricultural engineer still finds, among many farmers, a cautious attitude to the question; for this reason he endeavours to keep the tractor weight as low as possible. Laboratory experiments on soil suggest that the permissible range of moisture content suitable for tractor cultivation is not appreciably different from that for horse cultivation, provided the moisture is well distributed through the soil, and there is not a surface skin of wet soil. It is probable that most of the adverse reports about tractor pressures could be traced to some such circumstance.

COMPARISON OF DIFFERENT CULTIVATION METHODS.

The plough, cultivator, harrow, hoe, and roller form the existing range of implements used by the farmer to produce a tilth. The art of tilth production on different soils and in various weather conditions has been evolved by a long process of trial and error to a very high level, especially in this country. But it still remains an art; the soil scientist has not yet reduced it to a science. Hence, in many important directions the agricultural engineer is not provided with exact information on which to base his design of implements, and he has to rely on empirical observations. The art of cultivation is still based on cheap labour, and, owing to the

increased cost of work, it has for some time been by far the heaviest single item in the arable farmer's budget, and many attempts have been made to reduce these charges. The tractor is able to reduce cultivation costs provided it is given a full load, and this is definitely to the good. At the same time, various people have questioned whether the internal combustion engine could not be used in a more effective manner by applying its rotary motions direct to the soil—the process known as rotary cultivation—while others have critically examined the present range and sequence of the ordinary cultivation operations to see whether they could be reduced in number, or telescoped into combined operations without loss of efficiency. It will be convenient to examine these two ideas separately.

Rotary Cultivation.

The idea is not new. It was widely discussed nearly a century ago when steam power was first introduced for hauling cultivation implements, but although a number of rotary cultivation implements were introduced, they were unwieldy and did not, on the whole, do satisfactory work. The subject was reviewed when the internal combustion engine, with its higher ratio of horse-power to weight, became available. The small rotary cultivator, up to 10 h.p., controlled by a pair of stilt-handles, has for some years been used with success in market gardening and orchards. It has not yet been adopted for general agriculture on the medium and heavy lands that constitute the greater part of our arable area. For some years past experiments at Rothamsted have been done to examine this possibility. The great attraction of rotary cultivation is the claim that a tilth can be produced in one operation; the prevalent objection is that this tilth will be unsuitable because the rapidly revolving tines will produce too much breaking of the soil—the tilth will be too fine. The objection is quite understandable. The tines are attached to a revolving horizontal shaft at the rear of the machine, hence the tines revolve in a vertical plane. The tine points, after entering the soil, leave it in an upward direction. Some of the soil is carried violently upward into the cover built over the tool, and the whole depth of the stirred soil is subjected to what appears a very effective milling action. But actual experiments on the disruption or soil comminution produced, show that the objection is not really valid; rotary cultivation does not produce a much finer tilth than the traditional implements. The method* employed was to isolate with a spade and with the minimum disturbance, numerous blocks of soil, both before and after the passage of cultivation implements, and to pass these blocks through a set of sieves with apertures ranging from $1\frac{1}{2}$ in. to $\frac{1}{4}$ in. square. The weight remaining on each sieve was then expressed as a percentage of the total weight of the block. Typical results are shown in Fig. 1, which clearly demonstrates that the proportion of small sized pieces of soil produced by rotary cultivation is not excessive.

* B. A. Keen. *Empire Journal of Experimental Agriculture*, 1933, Vol. I, pp. 97-102.

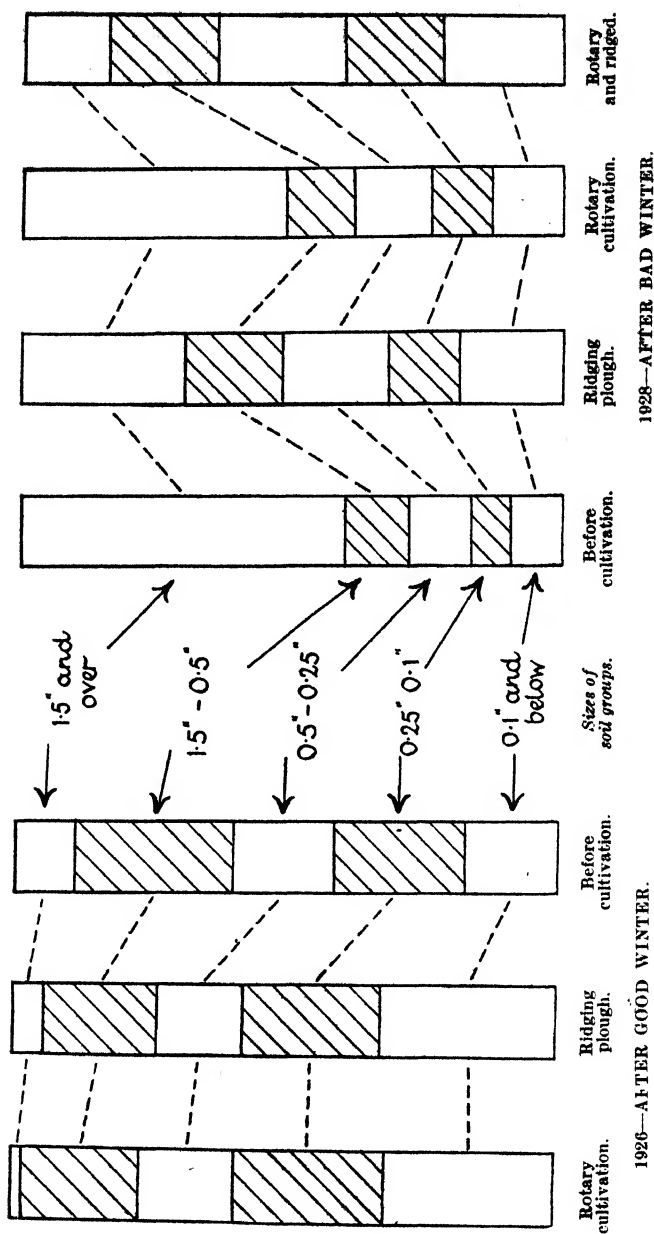


Fig. 1.—Percentage disintegration by sieving tests, showing effect of weather and cultivation on soil.

The real action of rotary cultivation is the production not of an unduly fine tilth, but of a loose or "fluffy" one. The upward movement of the tines loosens the whole soil, and greatly increases the pore space; a tine entry of 4 in. depth in the uncultivated soil produces a tilth that is at least 6 in. deep. The existing methods of seed-bed preparation and seed sowing are not adapted for use on such a loose tilth. It is this loose tilth and not its alleged fineness that constitutes the real problem awaiting solution before rotary cultivation can be widely used on medium and heavy land. The loose tilth has advantages and disadvantages. It encourages earlier germination and good root development; in all our experiments, with one exception, the early growth was superior on the rotary cultivated plots. But the tendency of a loose tilth to settle or consolidate under natural conditions, and to cap if rain falls, is a disadvantage, since it may injure the delicate root hairs of the

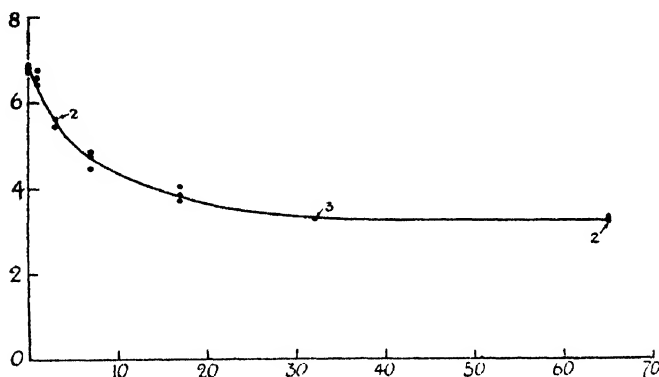


Fig. 2.—Consolidation, with time, of tilth on rotary-cultivated soil.

growing plant. The extent of settlement in Rothamsted soil can be seen from the results given in Fig. 2. It is possible that a light rolling, or, better, a subsoil packing immediately after the rotary cultivation, might be an improvement; the tools for this operation could be attached to the back of the rotary cultivator so that the whole process could be done in one operation.

A further point about rotary cultivation needs attention. The same tine action that causes the loose tilth also produces an appreciable mixing of the soil to the full depth of tine entry. In ordinary ploughing, the inversion of the furrow slice is more or less complete, and the seed-bed tilth is ultimately produced on the new top surface, whereas in rotary cultivation the tilth is in seed-bed condition throughout its whole depth. For this reason, and because the tines distribute the weed seeds throughout the whole depth of stirred soil, weeds germinate more strongly and in greater numbers than under the traditional cultivation methods. Further, in the traditional cultivation methods, the bulk of the weeds appear as seedlings

in the top layer of soil, and are, therefore, easily killed by hoeing, whereas some of the weeds on rotary cultivated soil may grow from well below the surface and are thus much more difficult to deal with. It has been suggested that the superior germination of weeds under rotary cultivation is an advantage, in that this process over several seasons will cause a permanent reduction in the viable weed-seed population of arable land. This point is now under investigation at Rothamsted as part of a long-range field experiment on different methods of soil cultivation.

It will thus be seen that rotary cultivation by tines that revolve in a vertical plane differs from traditional cultivation methods in the following three ways : a mixing of the different layers of soil, a loose tilth, and a distribution of weed seeds throughout the soil. It will be quite possible for the agricultural engineer and the farmer to devise for the remaining soil operations modified tools and methods which will operate satisfactorily on a rotary cultivated soil, should events necessitate it. But it will be wiser to make further study of rotary cultivation itself in the first instance. In this connexion the type of rotary cultivation performed by the Gyrotiller, manufactured by Messrs. Fowler under the Storey patents, deserves close attention. The Gyrotiller differs from other rotary instruments in that every part of the attached tine moves not in a vertical but a horizontal plane. An interesting detail of construction, which will be clear from the accompanying illustration (Fig. 3, Plate VI), is that the bars holding the tine points revolve around the surface of an imaginary truncated cone with a vertical axis. It should be possible to control the kind of cultivation by varying the shape of the bars. If they have no curvature, the soil would be stirred without much relative displacement—an action closely similar to that of the present-day cultivator. If the tine is curved throughout its whole length—having roughly the shape of a long narrow scoop—then the action should be somewhat like that of a plough with a sharply curved digger-breast, producing a combined inversion and mixing. If the tine is curved over its top part, and straight over the remainder of its length, then the action should be analogous to surface ploughing and sub-soiling. The essential feature of the Gyrotiller is that it avoids the violent upward lifting of the soil produced by other rotary cultivators, and thus produces not a loose tilth, but one more like that secured by the traditional methods. It remains to be seen whether gyrotillage or the other form of rotary tillage is the better for medium and heavy soils, and indeed whether rotary tillage will ultimately largely replace the traditional methods. To date, rotary tillage by small machines has secured a definite hold in the light land areas devoted to intensive farming and orchards. Its main use, apparently, is to prepare the land in which transplanting is to be done. This is an interesting point, since the two difficulties of a loose tilth and weed-seed distribution already mentioned, confronting the rotary cultivator for ordinary arable work, do not come in to such an extent. The transplanted plants are already a fair size and able to meet weed competition, while the loose tilth will be suitable for good root development, although around the plant itself the soil must be well

pressed down to give it a good hold in the loose tilth. With regard to heavy land, gyrotillage is being eagerly taken up by many farmers in place of contract cable ploughing by steam or Diesel sets. An exceedingly interesting development would be the production of a much smaller Gyrotiller attachment for the tractor.

The Study of Existing Cultivation Methods.

Rotary cultivation was introduced with the idea that it would be quicker and cheaper than the standard cultivation processes. One indirect result was to focus attention anew on the standard methods themselves to see whether the extensive operations that were practicable in days of cheap labour were a luxury or a necessity. For a long time all text-books have stressed the virtue of abundant cultivation in conserving moisture and raising soil fertility, and these views have coincided with the farmers' opinion. The question as to how far cultivation of the top surface to produce a dry mulch directly protects the soil moisture below from evaporation is fully discussed in the author's book.* It is sufficient here to state that numerous experiments show the mulch has probably no direct effect, and is only incidental to the operation of destroying weeds. There is a further opinion, widely held, that intensive inter-row cultivation of growing crops is of definite advantage to the yield. This point has been recently tested with surprising results. The trials were made with sugar beet on light soil and with kale and sugar beet on heavy land. In each case "ordinary" inter-row cultivation sufficient to keep down weeds was compared with "intensive" cultivation given on the average every ten days. The experiments were combined with a manurial trial also, so that the effects of the cultivations on differently manured plots could also be examined. The experiments were laid out in accordance with the statistical procedure devised at Rothamsted for field experiments, by which the degree of significance of the results can be estimated with precision. The results and the conclusions are given in the Appendix, Tables II, III, and IV.† It will be seen that in all cases a significant *reduction* in yield resulted from the intensive cultivations. Not only was the extra labour wasted, but the cash value of the crop was reduced. The experiments must be regarded as preliminary, and arrangements have been made for further trials over a number of seasons. However, they clearly show that the widely held opinion about the virtue of frequent surface cultivations requires adequate investigation. Even if it is subsequently shown that an increased yield is usually secured, the cash value of the increase must exceed the cost of the extra cultivations if the practice is to be justified.

THE PREDOMINANT EFFECT OF WEATHER ON TILTH.

There is no doubt that the farmer's close attention to cultivation methods arises from the care needed to obtain a satisfactory tilth in

* B. A. Keen. "The Physical Properties of the Soil" (Longmans, Green and Co.).

† Fuller details are given in the appropriate tables of the Rothamsted Report for 1932.

our variable weather conditions, on all soils, except the light sandy ones. The analysis of soil comminution by a nest of sieves, already referred to in this paper, provides a convenient means of demonstrating this point. Fig. 1 shows the results of spring cultivation of winter-ploughed soil after a "good" winter and after a "bad" one. The "good" winter was dry with spells of frost; the "bad" one was wet and mild. The condition of the soil after each winter is shown by the two columns marked "before cultivation." The soil after the good winter was so friable that even the gentle action of sieving was sufficient to reduce it to a good tilth; in this condition even the most rudimentary form of cultivation would suffice. The soil after the bad winter, on the other hand, consisted mainly of large unkindly lumps, the nature of which can be judged by the fact that the very drastic combined action of the rotary cultivator and the ridging or bouting plough was needed to comminute the soil to the same extent as was secured merely by passing the "good winter" soil gently over a sieve.

Since weather has such a predominant effect, it is clear that the farmer has to use every mechanical help possible, to get the soil in the right condition for the weather to act on it, and to counteract the deleterious effects of unsuitable weather. Therefore it is not surprising that he is keenly interested in all cultivation improvements the engineer can place before him, whether these be concerned with the tractor, the traditional cultivation implements, or new tools such as the rotary cultivator.

Finally, it should again be pointed out how little we really know of the underlying principles of soil cultivation. Our present methods have been evolved by slow empirical processes of trial and error. Although there are strong *a priori* reasons for assuming they are the best possible, there is no definite proof. We are, in fact, in the stage of turning an art into a science—a slow process, but an essential one, without which the soil physicist cannot supply the agricultural engineer with the information he needs.

NOTE.—The joint discussion on this paper and that by S. J. Wright (see page 195) is reproduced on pages 213–224.

APPENDIX.

GLOSSARY OF AGRICULTURAL TERMS.

- | | |
|----------------------------|--|
| Breast | see mouldboard. |
| Cable-cultivation | the cultivation implement is drawn backwards and forwards across the field by a cable which is wound on to a windlass carried on an engine on one side of the field and unwound from a similar windlass and engine on the opposite side. |
| Digger-breasted plough .. | a plough with a short and sharply curved mouldboard which only partially inverts the soil and leaves it rough and broken. |
| Grassland or sod plough .. | a plough with a long gently curving mouldboard, which will completely invert the soil and bury the grass. |

FUNCTIONS OF MECHANICAL POWER IN SOIL CULTIVATION. 191

Ley	grassland, as arable land laid down to grass for a period.
Mouldboard	the curved surface of the plough that turns over the earth in ploughing.
Ridging plough	a plough that throws up a ridge of soil on each side; cf. snow-plough.
Tilth	the condition in which the small soil particles are loosely aggregated into crumbs, or compound particles; this granular structure is spoken of as "good tilth," because in this condition it is most favourable for plant growth.

TABLE I.

COST PER ACRE FOR HORSE AND TRACTOR, INCLUDING WAGES.
(FIGURES FROM VARIOUS FARMS.)

Ploughing :	
Horse	20/-, 19/10, 14/10, 17/2
Tractor	15/9, 14/6, 11/11, 8/-
Cultivating :	
Horse	2/6, 4/-
Tractor	3/6, 4/5
Harrowing :	
Horse	1/6
Tractor	3/6
Rolling :	
Horse	1/6
Tractor	2/1
Harvest :	
Horse	2/7, 2/8, 2/1
Tractor	3/11, 3/6, 4/7½

TABLE II.

KALE—EFFECT OF THINNING, ORDINARY AND INTENSIVE CULTIVATION.
GREAT HARPENDEN, 1932.

YIELDS OF GREEN MATERIAL (EDGE ROWS EXCLUDED).

	Tons per acre.			Per cent.		
	Un-thinned.	Thinned.	Mean.	Un-thinned.	Thinned.	Mean.
Ordinary cultivation	27.65	25.18	26.42	108.5	98.8	103.6
Intensive cultivation	25.51	23.63	24.57	100.1	92.7	96.4
Mean	26.58	24.40	25.50	104.3	95.7	100.0

Standard error of single entry : 0.323 tons, or 1.27 per cent.

CONCLUSIONS.

Thinning and intensive cultivation both reduce the yield of green material significantly.

TABLE III.

SUGAR BEET. GREAT KNOTT, 1932.

	No nitro- gen.	Nitro- gen 3 weeks before sowing.	Nitro- gen at sowing.	$\frac{1}{2}$ N at sowing, $\frac{1}{2}$ N at sing- ling.	Mean of nitro- gens.	Mean.
Roots (washed), tons per acre.						
Ordinary cultivation :						
Early basal*.....	13.97	14.21	13.84	14.22	14.09	14.06
Late basal.....	12.95	14.04	14.44	14.17	14.22	13.90
Mean.....	13.46	14.12	14.14	14.19	14.15	13.98
Intensive inter-drill cultivation :						
Early basal.....	11.64	12.96	13.44	13.06	13.16	12.78
Late basal.....	12.22	13.48	13.93	12.88	13.43	13.13
Mean.....	11.93	13.22	13.68	12.96	13.29	12.95
Tops, tons per acre.						
Ordinary cultivation :						
Early basal.....	14.07	17.53	16.41	15.90	16.61	15.98
Late basal.....	12.92	17.82	17.07	15.19	16.70	15.75
Mean.....	13.50	17.68	16.74	15.54	16.66	15.86
Intensive inter-drill cultivation :						
Early basal.....	10.68	14.13	13.78	13.16	13.69	12.94
Late basal.....	11.96	13.90	14.88	13.95	14.24	13.67
Mean.....	11.32	14.02	14.33	13.56	13.97	13.30

Standard errors of single entries :—Roots : 0.276 tons, or 2.05 per cent.

Tops : 0.693 tons, or 4.75 per cent.

* " Basal " in this and later tables indicates that phosphatic and potassic manures were applied over the whole experimental area.

CONCLUSIONS.

Significantly greater yield with ordinary cultivation, both for roots (1.03 tons, or 7.6 per cent) and tops (2.56 tons, or 17.6 per cent). The difference in sugar percentage is small and not significant, being 0.06, or 0.4 per cent greater for ordinary cultivation.

FUNCTIONS OF MECHANICAL POWER IN SOIL CULTIVATION. 193

TABLE IV.

SUGAR BEET—WOBURN BUTT CLOSE, 1932.

	No nitro- gen.	Nitro- gen 3 weeks before sowing.	Nitro- gen at sowing.	$\frac{1}{2}$ N at sowing, $\frac{1}{2}$ N at sing- ling.	Mean of nitro- gens.	Mean.
Roots (washed), tons per acre.						
Ordinary cultivation :						
Early basal	11.37	12.04*	11.08	12.11	11.75	11.65
Late basal.....	10.72	11.89*	13.42	13.33	12.88	12.34
Mean.....	11.04	11.97	12.25	12.72	12.31	11.99
Intensive inter - drill cultivation :						
Early basal	11.22	11.76	13.12	11.87	12.25	11.99
Late basal.....	11.26	11.55	11.38	11.95	11.62	11.53
Mean.....	11.24	11.66	12.25	11.91	11.94	11.76
Tops, tons per acre.						
Ordinary cultivation :						
Early basal	13.32	15.70*	16.44	17.10	16.41	15.64
Late basal.....	12.73	16.24*	16.10	16.66	16.33	15.43
Mean.....	13.03	15.97	16.28	16.88	16.38	15.54
Intensive inter - drill cultivation :						
Early basal	12.91	17.54	17.06	16.56	17.05	16.02
Late basal.....	14.20	17.71	16.08	16.43	16.74	16.10
Mean.....	13.55	17.62	16.57	16.49	16.89	16.06

* The yield of one plot of each of these treatments was estimated.

Standard errors of single entries :—Roots : 0.415 tons, or 3.49 per cent.
Tops : 0.519 tons, or 3.29 per cent.

SUGAR PERCENTAGE.

	No nitro- gen.	Nitro- gen 3 weeks before sowing.	Nitro- gen at sowing.	$\frac{1}{2}$ N at sowing, $\frac{1}{2}$ N at sing- ling.	Mean of nitro- gens.	Mean.
Ordinary cultivation :						
Early basal	18.48	18.05*	18.08	18.10	18.08	18.18
Late basal	18.44	18.22*	18.22	17.97	18.14	18.21
Mean	18.46	18.14	18.15	18.04	18.11	18.20
Intensive inter - drill cultivation :						
Early basal	18.28	17.92	17.73	18.17	17.94	18.02
Late basal	18.21	17.68	17.96	18.07	17.90	17.98
Mean	18.24	17.80	17.84	18.12	17.92	18.00

* The yield of one plot of each of these treatments was estimated.

Standard error of single entry : 0.138, or 0.765 per cent.

CONCLUSIONS.

The higher yield of tops in the case of the inter-drill cultivation is not quite significant, and there is a non-significant reduction of 0.23 tons, or 2.0 per cent in the roots and a significant reduction of 0.20, or 1.1 per cent, in the sugar percentage by intensive cultivation.

THE EFFECTS OF RAINFALL AND TEMPERATURE ON PERCOLATION THROUGH DRAIN GAUGES.

By R. S. KOSHAL, M.Sc.,

IN CONSULTATION WITH R. A. FISHER, Sc.D., F.R.S.

(*Statistical Department, Rothamsted Experimental Station,
Harpenden, Herts.*)

(With Eight Text-figures.)

INTRODUCTION.

THE complexity of the physical factors controlling the quantity of water discharged by a drain gauge has been long recognised at Rothamsted. The three gauges containing respectively 20, 40 and 60 in. of soil have been installed since 1870. For the present study the 55 years, 1878–1932, have been taken. The object has been to ascertain to what extent the drainage in a month can be predicted from the recorded rainfall and temperature of the same month. It will appear that from 74 to 98 per cent. of the observed variance can be thus predicted, the precision of the predictions being greatest in the winter, doubtless because the gauges are most nearly saturated at this time of the year.

The enquiry would have been a relatively simple one but for the occurrence of slow secular changes, probably affecting the physical condition of the soil in the gauges. The existence of such changes was pointed out by Russell(2) in 1907. Russell especially noted the fact that these changes were not identical in the three gauges and that in this matter, as in some others, the three gauges, though in general remarkably similar in their discharge, showed notable individuality. In the present enquiry, therefore, allowance has been made for a steady change in the constants of the prediction formula. The formulae thus show (i) the expected percolation for each gauge under standard conditions for each month of the year, and the average rate of change of this quantity over the 55 years, (ii) the increment to be expected for each additional inch of rain in each month, and its average rate of change, and, finally, (iii) the difference to be expected for each additional degree of temperature, in the mean temperature of the month, and the average rate of secular change of this quantity.

In addition to these formulae, the residuals or deviations from the predicted values have been calculated and filed at Rothamsted. These, it is hoped, will form the basis of further enquiries, suggested by the many unexpected features presented by the data. In the present paper they have been used only to construct the series of percolation values, shown by Fig. 1 *a*, for the eleven periods of 5 years each, after correction for variation of temperature and rainfall, by which the absolute and relative values of the change in percolatability of the different gauges may be represented.

METHOD OF INVESTIGATION.

In considering the meteorological factors which may be expected to influence the amount of water percolating through the depth of soil in the drain gauge, attention is naturally directed towards the rainfall and the prevailing temperature. The object of the present enquiry is to ascertain to what extent the drainage recorded in a given calendar month can be expressed in terms of the rainfall and temperature of that same month. Previous meteorological conditions extending possibly for several months may be expected, of course, to influence the quantity and distribution of the water in the soil at the beginning of the month, but it would appear premature to attempt to discuss the nature or extent of this influence until more immediate meteorological factors have been considered.

Even with these the present investigation will be limited to the average response to unit variations in the rainfall, or the temperature. It would be unreasonable to suppose that the response in drainage to a given inch of rain will be the same in a wet as in a dry month, consequently it is to be anticipated that a more elaborate non-linear formula in terms of rainfall, and possibly also in terms of temperature would give a closer prediction. It is a principal object of the present enquiry to provide material for such further studies. Nevertheless, it is believed that the simple average response to increments of rainfall and temperature, and the comparison of these averages for the different gauges and the different months of the year, will always be of more immediate interest than effects of higher order. We shall consequently study the possibilities of a prediction formula of the simple form

$$P = c + b_2 R + b_4 T, \quad \dots\dots(1)$$

where c , b_2 and b_4 are constants for a given gauge in a given month of the year, and R and T stand for the total rainfall, and the monthly mean temperature derived from the readings of the maximum and

minimum thermometers. Air temperatures only are available, but the variations in the monthly averages of these will certainly correspond closely to those of the mean temperature in the soil mass.

In the study of these historical gauges a further most important factor must be taken into account. As was to be expected and has long been recognised by writers on these gauges, slow changes have been going on in the condition of the soil of a kind which might well influence both the average amount of water percolating and the average response to increments of rainfall and temperature. We must therefore take time into account as an independent variate. As in the case of the other variates we shall consider only the average rate of change during the 55 years. Even this, however, leads to the introduction of three new terms. The form of regression equation finally adopted is therefore

$$P = c + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4 + b_5x_5, \quad \dots\dots(2)$$

where x_1 stands for the date measured from 1900, x_2 for the rainfall measured from zero, x_3 for the product of x_1 and x_2 , x_4 for the temperature in degrees F. from an arbitrary origin T_m chosen to suit convenience for each month, and x_5 is the product of x_3 and x_4 . The purpose of choosing variates x_3 and x_5 , each of which is the product of two of the other variates used, is to provide values not merely for the regressions of percolation upon rainfall and temperature, but for the rates of change of these regression coefficients.

For, if we substitute in this formula, which is given in terms of the variates most easy to handle arithmetically, the variates in which the results are most simply interpreted, namely

$$x_1 = t = t' + 5,$$

where t' is the time measured from 1905, the central year of the period investigated,

$$x_2 = R = \bar{R} + R',$$

where R' is the deviation of the rainfall from its average value for the month considered, then

$$x_3 = tR = (t' + 5) (\bar{R} + R').$$

$$\text{Again} \quad x_4 = T - T_m = (\bar{T} - T_m) + (T - \bar{T}) = (\bar{T} - T_m) + T',$$

where T' is the deviation of the temperature of the month from its mean value, and consequently

$$x_5 = t (T - T_m) = (t' + 5) (\bar{T} - T_m + T').$$

108 *Effects of Rainfall and Temperature on Drainage*

Making these substitutions in equation (2) we have

$$P = c + b_1(t' + 5) + b_2(\bar{R} + R') + b_3(t' + 5)(\bar{R} + R') \\ + b_4(\bar{T} - T_m + T') + b_5(t' + 5)(\bar{T} - T_m + T'),$$

or, more simply,

$$P = c + 5b_1 + b_2\bar{R} + 5b_3\bar{R} + b_4(\bar{T} - T_m) + 5b_5(\bar{T} - T_m) \\ + t'(b_1 + b_3\bar{R} + b_5(\bar{T} - T_m)) \\ + R'(b_2 + 5b_3) + T'(b_4 + 5b_5) + b_3t'R' + b_5t'T',$$

which may be written

$$P = c' + b_1't' + b_2'R' + b_3't'R' + b_4'T' + b_5't'T',$$

where

$$b_3' = b_3,$$

$$b_5' = b_5,$$

$$b_2' = b_2 + 5b_3,$$

$$b_4' = b_4 + 5b_5,$$

$$b_1' = b_1 + b_3\bar{R} + b_5(\bar{T} - T_m),$$

$$c' = c + 5b_1 + b_2\bar{R} + 5b_3\bar{R} + b_4(\bar{T} - T_m) + 5b_5(\bar{T} - T_m).$$

The regression formula may now be written in the simple form

$$P = (c' + b_1't') + (b_2' + b_3't') R' + (b_4' + b_5't') T', \quad \dots\dots(3)$$

showing that we have obtained a simple regression equation in the two variates R' and T' representing the deviations of the observed rainfall and temperature from the average of the month in question, with the added feature that the three constants of the formula are now each of them linear functions of the time capable of representing any steady change in the values of these constants.

Returning now to equation (2) the best values of b_1 , b_2 , b_3 , b_4 and b_5 are obtained from the solution of partial regression equations. Dr Fisher's "matrix method" has been employed for the solution of these equations, since it is not only the quickest method (especially in the present case, where we have three dependent variates P_1 , P_2 , P_3 indicating drainage through the 20, 40 and 60 in. gauges respectively, each of which has the same set of independent variates) of evaluating partial regression coefficients, but it also provides material for the calculation of their standard errors. The method of solution and substitution is given in Dr Fisher's *Statistical Methods for Research Workers*, Section 29(1). The advantages of the indirect method of solution there given for work involving many independent variates will be more readily appreciated if the procedure of successive substitution used to obtain the values of c_{12} , c_{13} , etc., from c_{11} be described in detail. The month of June has been chosen for this purpose.

The numerical values of the fifteen sums of squares and products of deviations of x_1, x_2, x_3, x_4 and x_5 from their respective means form the coefficients in the five equations of least squares:

$$\begin{aligned} b_1 S(x_1^2) + b_2 S(x_1 x_2) + b_3 S(x_1 x_3) + b_4 S(x_1 x_4) + b_5 S(x_1 x_5) &= S(x_1 P), \\ b_1 S(x_1 x_2) + b_2 S(x_2^2) + b_3 S(x_2 x_3) + b_4 S(x_2 x_4) + b_5 S(x_2 x_5) &= S(x_2 P), \\ &\text{etc.,} \end{aligned}$$

by which the best values for $b_1 \dots b_5$ may be obtained for each of the drain gauges, if its readings are substituted in the sums of products on the right of the equations. Instead of solving these equations as they stand, however, it is always preferable, when standard errors are required, to invert the matrix of coefficients by carrying out the arithmetical solution on the equations in which the right-hand side is either unity or zero. The equations required for the month of June are:

$$\left. \begin{aligned} +13860.000b_1 - 309.920b_2 + 27037.110b_3 - 255.000b_4 + 26020.200b_5 &= 1, 0, 0, 0, 0 \\ - 309.920b_1 + 88.002b_2 - 438.672b_3 - 35.081b_4 - 651.284b_5 &= 0, 1, 0, 0, 0 \\ + 27037.110b_1 - 438.672b_2 + 73350.575b_3 - 559.708b_4 + 41755.414b_5 &= 0, 0, 1, 0, 0 \\ - 255.000b_1 - 35.081b_2 - 559.708b_3 + 169.899b_4 + 719.889b_5 &= 0, 0, 0, 1, 0 \\ + 26020.200b_1 - 651.284b_2 + 41755.414b_3 + 719.889b_4 + 88516.997b_5 &= 0, 0, 0, 0, 1 \end{aligned} \right\}, (i)$$

where the left-hand side is identical with that of the equations of least squares. The values of $b_1 \dots b_5$ obtained by such sets of equations are denoted by:

c_{11}	c_{12}	c_{13}	c_{14}	c_{15}
c_{12}	c_{22}	c_{23}	c_{24}	c_{25}
c_{13}	c_{23}	c_{33}	c_{34}	c_{35}
c_{14}	c_{24}	c_{34}	c_{44}	c_{45}
c_{15}	c_{25}	c_{35}	c_{45}	c_{55}

which constitutes the inverted matrix.

In order to obtain the first row or column of this matrix we substitute c_{11}, c_{12}, \dots for b_1, b_2, \dots in the first set of equations (i), and eliminating c_{15} from the first four by cross multiplication we have:

$$\left. \begin{aligned} + 5497.948c_{11} - 104.8665c_{12} + 13067.596c_{13} - 413.0349c_{14} &= 0.88516997 \\ - 104.8665c_{11} + 73.65502c_{12} - 116.35295c_{13} - 26.36413c_{14} &= 0 \\ + 13067.596c_{11} - 116.35295c_{12} + 47492.580c_{13} - 796.0293c_{14} &= 0 \\ - 413.0349c_{11} - 26.36413c_{12} - 796.0293c_{13} + 145.2070c_{14} &= 0 \end{aligned} \right\} \dots (ii)$$

From these, using the fourth equation to eliminate c_{14} from the first three by cross multiplication, we have:

$$\left. \begin{aligned} + 6277.427c_{11} - 261.1664c_{12} + 15687.1853c_{13} &= 1.28532876 \\ - 261.1664c_{11} + 100.00157c_{12} - 378.81883c_{13} &= 0 \\ + 15687.1853c_{11} - 378.81883c_{12} + 62625.924c_{13} &= 0 \end{aligned} \right\} \dots (iii)$$

110 *Effects of Rainfall and Temperature on Drainage*

Now using the third equation to eliminate c_{13} from the first two, we have:

$$\left. \begin{aligned} +1470.4188c_{11} - 104.13186c_{13} &= 0.804949012 \\ -104.13186c_{11} + 61.191870c_{13} &= 0 \end{aligned} \right\}, \quad \dots (iv)$$

from which the value of c_{11} is at once obtained:

$$\begin{aligned} 791.342318c_{11} &= 0.492563353, \\ \text{or} \quad c_{11} &= 0.0006224403. \end{aligned}$$

The value of c_{12} is now obtained by substitution in the second equation of (iv) and its accuracy is checked by recalculating the value of c_{11} from the first equation. The value of c_{13} is computed from the last equation of (iii); c_{12} and c_{11} are then recalculated from the second and first equations respectively. The process is continued until c_{15} is evaluated and checked by at least one substitution. In this manner c_{11} is checked four times, c_{12} thrice, c_{13} twice and c_{14} once only. The values obtained by the complete process of substitution are given below to show the degree of concordance to be expected, when as above not less than seven figures are retained in the terms of the leading diagonal in the process of elimination.

c_{11}	c_{12}	c_{13}	c_{14}	c_{15}
0.0006224403	—	—	—	—
0.0006224403	0.0010592234	—	—	—
0.0006224403	0.0010592235	-0.0001495081	—	—
0.0006224403	0.0010592236	-0.0001495081	0.0011432107	—
0.0006224403	0.0010592236	-0.0001495081	0.0011432103	-0.0001139485

This provides the values for the first column or row of the matrix, and these values are fixed and are not altered by the further process of substitution. By solving only one set of equations, we are now in a position (and this is a most important point of the matrix method) to obtain the solution of the other four equations, and through them of any system of equations with the same matrix of coefficients, by the methodical process of substitution only. Thus the values of c_{22} , c_{23} , c_{24} and c_{25} (which together with c_{12} form the second row or column of the matrix) are obtained from the solution of the equations (iv) to (i) by writing c_{12} , c_{22} , c_{23} , c_{24} and c_{25} in place of c_{11} , c_{12} , c_{13} , c_{14} and c_{15} and assigning the non-vanishing element of the right-hand side to the second equation of each group instead of to the first. The first equation of each group is now ignored. Equation (iv) becomes:

$$-104.13186c_{12} + 61.19187c_{22} = 0.804949012, \quad \dots (iv')$$

from which, knowing c_{12} , c_{22} is immediately evaluated. c_{23} is next obtained from the third equation of (iii') and its accuracy is checked by recalculating

lating c_{22} from the second equation, c_{24} and c_{25} are computed in a similar manner from the equations (ii') and (i'). The values of c_{22} , c_{23} , c_{24} and c_{25} are now fixed and are not further altered in the evaluation of other values of c .

The values of c_{33} , c_{34} and c_{35} are now obtained from the equations (iii), (ii) and (i) by a similar process of elimination and checking, by assigning the non-vanishing element of the right-hand side to the third equation of each group and ignoring the first *two* equations of each group. c_{44} and c_{45} are obtained from (ii) and (i), and finally c_{55} is obtained from (i) by a similar process.

The complete matrix thus evaluated is given below in units of 10^{-4} .

$$\begin{aligned}
 &+ 6.224403 + 10.592236 - 1.495081 + 11.432103 - 1.139485 \\
 &+ 10.592236 + 149.570185 - 1.748515 + 47.700023 - 1.576288 \\
 &- 1.495081 - 1.748515 + 0.569165 - 1.449974 + 0.169929 \\
 &+ 11.432103 + 47.700023 - 1.449274 + 94.189000 - 3.091617 \\
 &- 1.139485 - 1.576288 + 0.169929 - 3.091617 + 0.381319
 \end{aligned}$$

This matrix is used for a number of essential purposes, some of which we shall now discuss.

(a) Calculation of partial regression coefficients.

The regression coefficients being the solutions of equations having on the right-hand side the sums of products $S(Px_1)$, etc. (where P now indicates the deviation from the mean P) are obtained from the equations:

$$\begin{aligned}
 b_1 &= S(Px_1)c_{11} + S(Px_2)c_{12} + S(Px_3)c_{13} + S(Px_4)c_{14} + S(Px_5)c_{15}, \\
 b_2 &= S(Px_1)c_{12} + S(Px_2)c_{22} + S(Px_3)c_{23} + S(Px_4)c_{24} + S(Px_5)c_{25}, \\
 &\text{etc.}
 \end{aligned}$$

Hence the appropriate equations for the three different gauges are obtained merely by inserting in three linear equations the sums of products appropriate to each gauge. The values of these for the three gauges for the month of June are:

	P_1	P_2	P_3
$S(Px_1)$	-161.741	-155.991	-147.861
$S(Px_2)$	+ 49.365	+ 48.800	+ 48.115
$S(Px_3)$	-284.809	-272.203	-251.176
$S(Px_4)$	- 22.491	- 20.778	- 20.682
$S(Px_5)$	-301.957	-315.899	-295.740

The value of b_1 for the 20 in. gauge is obtained by multiplying the sums of products for P_1 in order with the elements of the first row or column of the matrix and adding; b_2 is obtained by multiplying the same sums of products with the elements of the second row or column and

112 *Effects of Rainfall and Temperature on Drainage*

adding. In a similar manner b_3 , b_4 and b_5 are obtained from the third, fourth and fifth rows or columns of the matrix. Using the sums of products for P_2 and P_3 , the partial regression coefficients for the 40 and 60 in. gauges can be calculated. Thus from the matrix these coefficients can be computed immediately, and the special advantage and quickness of the method lies in the fact that the same matrix can be used for any number of dependent variates (having the same set of independent variates), consequently the labour of solving the regression equations afresh for each new dependent variate is saved. The values of these coefficients for the month of June are:

	P_1	P_2	P_3
b_1	+0.00289131	+0.00753417	+0.00653799
b_2	+0.55714761	+0.56295193	+0.55492154
b_3	-0.00253027	-0.00305896	-0.00262927
b_4	-0.02662319	-0.00412771	-0.00647790
b_5	+0.00124823	-0.00016493	+0.00011297

(b) *Calculation of standard errors of partial regression coefficients.*

An essential point of the matrix method is that it provides ready material for the calculation of standard errors. The sampling variance of the regression coefficient is obtained by multiplying the residual variance with the appropriate matrix element. The standard error is obtained from the sampling variance by taking the square root. Thus the standard error of b_1 is $s\sqrt{c_{11}}$, of b_2 is $s\sqrt{c_{22}}$, etc., where s^2 is the residual variance.

The sum of the squares of deviations from the regression equation is obtained from the sum of squares of deviations from the mean by deducting a term for each coefficient fitted:

$$S(P - P')^2 = S(P^2) - b_1 S(x_1 P) - b_2 S(x_2 P) - b_3 S(x_3 P) - b_4 S(x_4 P) - b_5 S(x_5 P).$$

The only new value to be computed is $S(P^2)$, and the values of the various terms to be deducted are obtained by multiplying b_1, b_2, \dots, b_5 , with the corresponding sums of products $S(P_1 x_1), S(P_1 x_2), \dots$ etc., given in § (a). The residual variance s^2 is obtained by dividing $S(P - P')^2$ by the appropriate degrees of freedom (49 in the present case). By multiplying the value of s^2 with the five principal diagonal elements of the matrix we at once obtain the values of the variances of the five regression coefficients. Using the same set of multipliers and s^2 for the 40 and 60 in. gauges, the standard errors of the regression coefficients for these two gauges can be calculated. Thus the standard errors of the regression

coefficients for any number of dependent variates can be immediately calculated from the matrix. The numerical values of the quantities $S(P^2)$, $S(P-P')^2$, s^2 , and the standard errors of the regression coefficients given in (a) are:

	P_1	P_2	P_3
$S(P^2)$	39.231	37.998	37.589
$S(P-P')^2$	11.261	10.730	11.095
s^2	0.229816	0.218980	0.226428
$\sigma_{b_1} = \sqrt{s^2 c_{11}}$	0.0119598	0.0116754	0.0118720
$\sigma_{b_2} = \sqrt{s^2 c_{22}}$	0.0586260	0.0572318	0.0581955
$\sigma_{b_3} = \sqrt{s^2 c_{33}}$	0.0036165	0.0035305	0.0035899
$\sigma_{b_4} = \sqrt{s^2 c_{44}}$	0.0465233	0.0454169	0.0461817
$\sigma_{b_5} = \sqrt{s^2 c_{55}}$	0.0029601	0.0028897	0.0029384

A complete set of the values of s^2 for the twelve calendar months and for the three gauges is given in Table X.

(c) *Calculation of standard errors of b_2' and b_4' .*

b_2' and b_4' indicate the values of b_2 and b_4 at the central date 1905, and as has already been indicated they are obtained from the equations

$$b_2' = b_2 + 5b_3; \quad b_4' = b_4 + 5b_5.$$

The variances of these quantities are given by:

$$\sigma_{b_2'}^2 = s^2 (c_{22} + 10c_{23} + 25c_{33}),$$

$$\sigma_{b_4'}^2 = s^2 (c_{44} + 10c_{45} + 25c_{55}),$$

where s^2 is the residual variance. Thus the matrix provides a ready solution for the calculation of the standard errors of these quantities, or of any other quantity which is connected with the values of the b 's by a linear equation. The numerical values of b_2' , b_4' with their standard errors for the three gauges for the month of June are:

	P_1	P_2	P_3
b_2'	+ 0.54450 \pm 0.05800	+ 0.54770 \pm 0.05660	+ 0.54177 \pm 0.05756
b_4'	- 0.02037 \pm 0.04190	- 0.00493 \pm 0.03993	- 0.00593 \pm 0.04059

The values of b_2' , b_4' with the associated standard errors for all the months are given in Tables VI and VIII.

(d) *Calculation of standard errors of the difference between regression coefficients for the different gauges.*

One of the most important uses to which the matrix can be put is to calculate the standard error of the difference of two regression coefficients obtained from two dependent variates (having the same set of

114 *Effects of Rainfall and Temperature on Drainage*

independent variates), allowing for the fact that the two sets of residuals from the two regression equations may be mutually highly correlated. The only new quantity to be evaluated for this purpose is the *residual covariance*, which is computed from the sums of products of the two dependent variates in the same manner as the residual variance is obtained from the sum of squares of each of them.

The sum of products of the deviations from the regression equations for the two gauges P_1 and P_2 is obtained by deducting from the sum of the products of the deviations a term for each coefficient fitted:

$$\begin{aligned} S(P_1 - P_1')(P_2 - P_2') &= S(P_1 P_2) - b_{1P_1} S(P_2 x_1) - b_{2P_1} S(P_2 x_2) \\ &\quad - b_{3P_1} S(P_2 x_3) - b_{4P_1} S(P_2 x_4) - b_{5P_1} S(P_2 x_5) \\ &= S(P_1 P_2) - b_{1P_2} S(P_1 x_1) - b_{2P_2} S(P_1 x_2) \\ &\quad - b_{3P_2} S(P_1 x_3) - b_{4P_2} S(P_1 x_4) - b_{5P_2} S(P_1 x_5), \end{aligned}$$

where b_{P_1} , b_{P_2} indicate the values of the regression coefficients for the 20 and 40 in. gauges. The values of these terms are immediately obtained by cross-multiplication—*i.e.* by multiplying the value of b 's of one gauge with the sums of products $S(Px)$ of the other, given in § (a). Since the two equations must give identical results, this process provides a check on the arithmetic of the calculation of regression coefficients for the 20 and 40 in. gauges from the matrix. In the same manner we can calculate the values of $S(P_1 - P_1')(P_3 - P_3')$ and $S(P_2 - P_2')(P_3 - P_3')$ and thus the values of all the regression coefficients are checked. The values of residual covariance for the different pairs of gauges are obtained by dividing these sums of products by the appropriate degrees of freedom (49 in the present case).

From the values of residual variance and covariance a quantity ψ^2 is computed from the equation which is merely the sum of the two residual variances minus twice the residual covariance, and represents the residual variance of the difference between the two gauge readings:

$$\psi^2_{P_1 P_2} = s_1^2 + s_2^2 - 2p_{12},$$

where s_1^2 , s_2^2 are the residual variances for the 20 and 40 in. gauges and p_{12} is the residual covariance between them. In a similar manner $\psi^2_{P_1 P_3}$ and $\psi^2_{P_2 P_3}$ are computed. A complete set of the values of ψ^2 for all the calendar months is given in Table XI. The standard error of the difference of the regression coefficients can now at once be obtained by multiplying ψ^2 with the appropriate matrix element and taking the square root of the product. Thus the standard error of $b_{2P_1} - b_{2P_2}$ is $\sqrt{\psi_{P_1 P_2} c_{22}}$, and of

$b_{4P_1} - b_{4P_2}$ is $\sqrt{\psi^2_{P_1P_2} c_{44}}$. Their values for the month of June for the three gauges are:

	$P_1 - P_2$	$P_2 - P_3$	$P_1 - P_3$
b_{2P_1}	-0.00580 ± 0.00971	$+0.00803 \pm 0.00491$	$+0.00223 \pm 0.00863$
b_{4P_1}	-0.02249 ± 0.00771	$+0.00235 \pm 0.00390$	-0.02014 ± 0.00685

The standard errors for the difference of two b_2' or two b_4' can be obtained by multiplying the ψ^2 with the appropriate expression involving matrix elements, and taking the square root of the resulting product.

Thus

$$\sigma_{(b_2'_{P_1} - b_2'_{P_2})} = \sqrt{\{\psi^2_{P_1P_2} (c_{22} + 10c_{23} + 25c_{33})\}},$$

$$\sigma_{(b_4'_{P_1} - b_4'_{P_2})} = \sqrt{\{\psi^2_{P_1P_2} (c_{44} + 10c_{45} + 25c_{55})\}}.$$

Thus the matrix provides solution for the standard errors of the difference of two regression coefficients each one of which is connected with the b 's by a linear relation of any form.

The differences between the regression coefficients with their standard errors (for b_2' and b_4') for the twelve calendar months and for the three gauges are given in Tables VI and VIII.

DISCUSSION OF RESULTS.

Each month has been treated in exactly the same manner as the month of June described in detail in the preceding section. The results obtained from this preliminary analysis for all the months are classified

Table I. *Mean monthly values of rainfall, temperature and drainage.*

Month	Rainfall R	Temperature		Drainage		
		\overline{T}	(T_m)	20 in. gaugo (P_1)	40 in. gaugo (P_2)	60 in. gaugo (P_3)
Jan.	2.302	37.547	(0)	1.9361	2.1139	2.0411
Feb.	2.025	38.436	(30)	1.5637	1.6728	1.6099
Mar.	1.987	41.049	(35)	1.0877	1.2123	1.1565
Apr.	2.046	45.384	(40)	0.6868	0.7655	0.7325
May	2.130	51.898	(50)	0.5517	0.6250	0.5923
June	2.164	57.076	(55)	0.5451	0.5819	0.5645
July	2.679	60.524	(55)	0.7155	0.7432	0.7034
Aug.	2.735	59.871	(55)	0.7964	0.8123	0.7669
Sept.	2.284	55.685	(50)	0.8052	0.8021	0.7501
Oct.	3.121	48.625	(40)	1.8207	1.8143	1.7080
Nov.	2.846	41.905	(35)	2.1949	2.2579	2.1576
Dec.	2.850	38.575	(30)	2.4593	2.5580	2.4768
Total: Oct.-Mar.	15.131	—	—	11.0624	11.6292	11.1499
Total: Apr.-Sept.	14.038	—	—	4.1007	4.3300	4.1097
Total: 12 months	29.169	—	—	15.1631	15.9592	15.2596
Mean	2.431	48.048	—	1.2636	1.3300	1.2550

MEAN MONTHLY VALUES

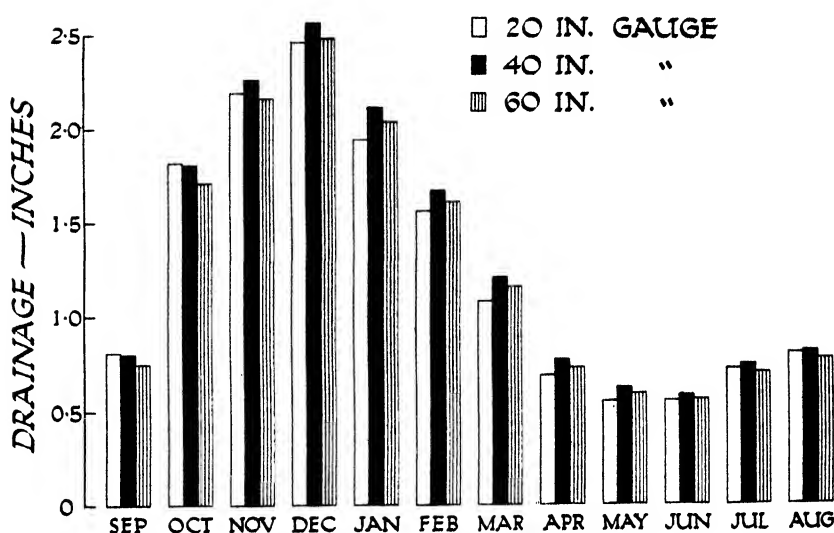


Fig. 1.

AVERAGE ANNUAL PERCOLATION IN 5-YEAR PERIODS (AFTER ALLOWANCE FOR VARIATIONS OF RAINFALL AND TEMPERATURE)

□ 20 IN. GAUGE ■ 40 IN. GAUGE ▨ 60 IN. GAUGE

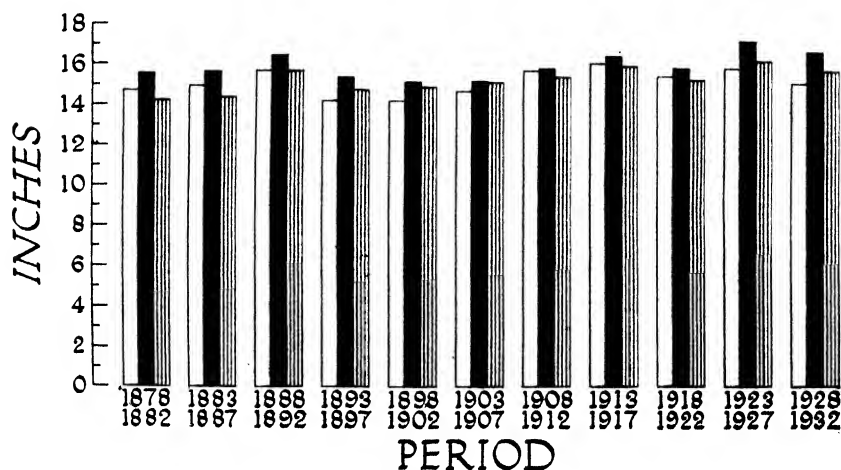


Fig. 1 a.

in Tables I–XIII, and are graphically illustrated in Figs. 1–8. We shall now consider the more salient features of these tables and diagrams.

Table I gives the mean monthly values of rainfall, temperature and drainage for the 55 years. Unlike temperature and drainage, the mean rainfall values do not differ much in the winter and summer. Of the winter months November and December are associated with heavy rainfall, January and February with low rainfall. In summer July and August have given high values of mean rainfall (like November and December), as compared with May and June which are as dry as January and February. The temperature values have shown remarkable regularity; they slowly rise from January to July and then gradually fall off to December. The figures shown in brackets are the values of T_m used for different months. The difference in average percolation between the warmer and the cooler months can be seen from the fact that the mean monthly rainfall from February to June is practically the same (about 2 in.), but the drainage in June is about half that of March and about a third of that of February. This is also evident from the six-monthly totals—October to March, April to September—which are practically the same for the rainfall, but the drainage of the former is about three times that of the latter.

It has been customary, and natural, to ascribe this difference to a much greater evaporation in the warmer than in the cooler months. This inference can, however, only be drawn by neglecting the variations, which are not directly observable, in the water content of the gauges. Although over a long period of years the total evaporation can be fairly estimated from the difference between the rainfall and the percolation, it should be emphasised that the distribution of evaporation over the year is not known. It will appear in Table III that when allowance is made for difference in temperature, the distribution of percolation over the year is but little altered. Further, in Table VIII it will be shown that comparing the same month in different years the effect of higher temperature is not regularly to decrease percolation, but on the whole to increase it. The inference that the smaller percolation of the summer months may be ascribed to an immediate increase in evaporation due to higher temperature is therefore questionable. The higher drainage in the cold months of winter must be at least partly accounted for by the accumulation of water during the preceding months of heavy rainfall, and the lower drainage in summer by the corresponding loss of water in the gauges during the preceding months of low rainfall.

The mean monthly drainage values for the three gauges are shown

118 *Effects of Rainfall and Temperature on Drainage*

in Fig. 1 in the form of columns of appropriate height, one for each gauge. The 40 in. gauge has given higher mean drainage in all the months except the first two (September and October) than the neighbouring 20 or 60 in. gauge. Considering the 20 and 60 in. gauges only, we find that the 60 in. gauge has continually given higher mean drainage than the 20 in. gauge from December to June, while the reverse is the case from July to November.

The average annual percolation after allowance for variations of rainfall and temperature is shown for eleven 5-year periods in Fig. 1 *a*. This was constructed by applying to the mean rates of secular increase in drainage the deviations of the actual drainage recorded from that predicted from the rainfall and temperature. These deviations are individually small, but their totals show secular changes of remarkable consistency. They are predominantly positive for the first 15 years, predominantly negative for the next 15 years and for the last 25 years fluctuate somewhat irregularly. The secular changes which have been taking place in the percolatability of the three gauges are not great in absolute magnitude, but appear to be due to real changes in the condition of the soil, and, though the parallelism between the three gauges is, throughout the data, remarkably close, yet the consistent changes to which each is subject appear to be somewhat different. The diagram shows, for example, considerable consistency in the changes taking place in the differences between the percolations through the 20 and 60 in. gauges.

Table II. *Variance of rainfall, temperature and drainage.*

Month	Rainfall (<i>R</i>)	Temperature (<i>T</i>)	Drainage		
			20 in. gauge (<i>P</i> ₁)	40 in. gauge (<i>P</i> ₂)	60 in. gauge (<i>P</i> ₃)
Jan.	1.01836	10.925	1.048740	1.239221	1.113851
Feb.	1.74970	13.246	1.911851	1.961407	1.866351
Mar.	1.30951	6.295	1.282258	1.440166	1.321703
Apr.	1.06629	3.672	0.634333	0.672740	0.609611
May	1.17914	4.015	0.511499	0.563407	0.473982
June	1.62963	3.146	0.726500	0.703667	0.696092
July	2.09499	5.549	0.633222	0.672981	0.605815
Aug.	2.15692	4.157	1.191555	1.187259	1.081111
Sept.	2.07156	4.195	1.225203	1.288425	1.230166
Oct.	2.56493	6.436	2.152610	2.180554	2.011629
Nov.	1.57626	6.206	1.566037	1.713018	1.606555
Dec.	2.56809	10.350	2.755406	2.661222	2.520259

Table II gives the variance of monthly values of rainfall, temperature and drainage for the 55 years. Like the mean values, the rainfall variance shows higher values in autumn than in spring; its value is small from

January to June, and comparatively greater from July to December (with the exception of November). The temperature variance is much greater in the winter months December, January and February, as compared with other months of the year, the highest value in February being about four times the lowest value in June. It is interesting to note that there is a remarkable oscillation in the rainfall variance, particularly in the winter months which is reflected in the variances of the drainage shown diagrammatically in Fig. 2. Comparing the three gauges

VARIANCE IN DRAINAGE

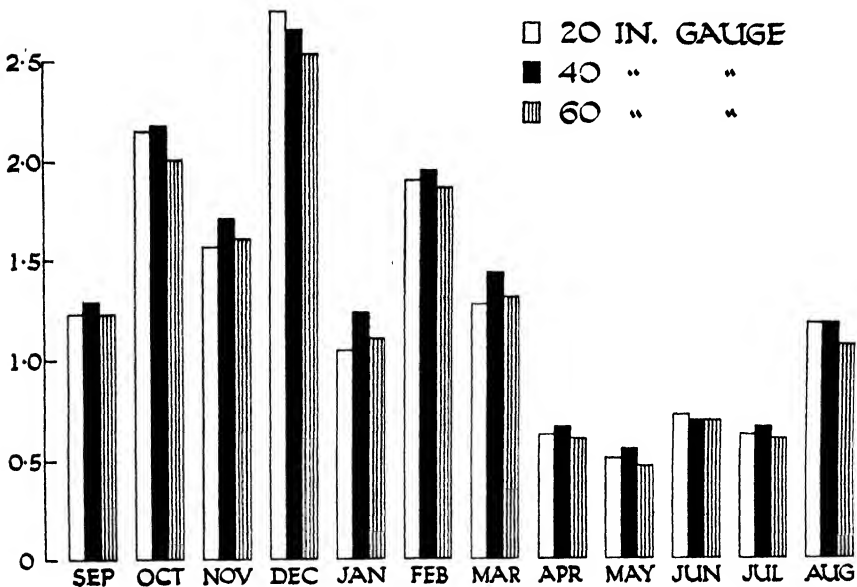


Fig. 2.

we find that the 40 in. gauge has given greater variability than either the 20 or 60 in. gauge. Considering the extreme gauges 20 and 60 in. only, we find that, from September to March, the 60 in. gauge has shown greater variability than the 20 in. gauge in alternate months, while from April to August the 20 in. gauge has shown continuously greater variability than the 60 in. gauge. A distinct seasonal effect exists in the drainage variance values, April to September having yielded exceptionally low values as compared with other months, a fact which can clearly be seen from Fig. 2, and corresponds with the lower mean drainage.

120 *Effects of Rainfall and Temperature on Drainage*

Table III and Fig. 3 give the values of drainage under standard conditions, the standard for rainfall adopted is approximately the mean

DRAINAGE IN STANDARD CONDITIONS 2.5 INS. RAIN, 48° F.

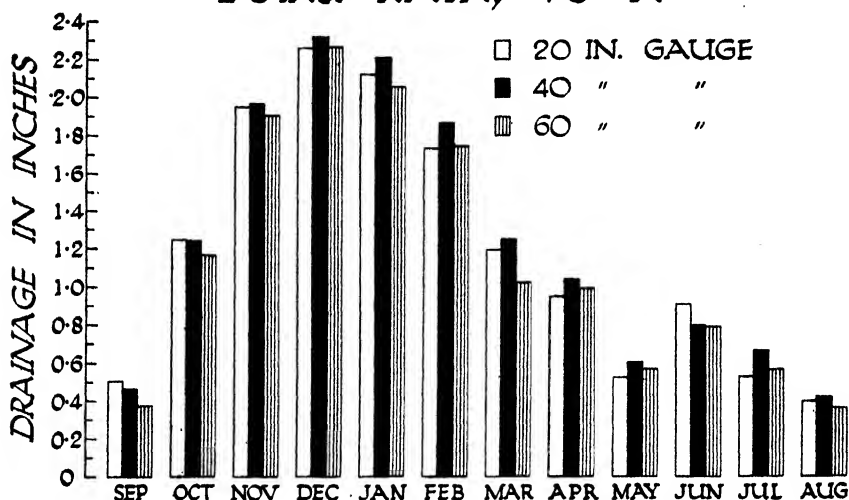


Fig. 3.

monthly rainfall for 55 years, 2.5 in., and the mean monthly temperature, 48° F. The standardised drainage has been obtained from the regression equations of form (2) for different months using the value of $x_1 = 5$, so that the values calculated are those expected for the year 1905,

Table III. *Drainage under standard conditions:
rainfall 2.5 in., temperature 48° F.*

Month	20 in. gauge P_1	40 in. gauge P_2	60 in. gauge P_3
Jan.	2.120	2.210	2.057
Feb.	1.734	1.863	1.742
Mar.	1.196	1.256	1.229
Apr.	0.953	1.021	0.988
May	0.531	0.605	0.576
June	0.904	0.793	0.786
July	0.536	0.641	0.567
Aug.	0.399	0.422	0.365
Sept.	0.509	0.469	0.380
Oct.	1.246	1.244	1.164
Nov.	1.948	1.965	1.912
Dec.	2.264	2.320	2.268
Total: Oct.-Mar.	10.508	10.858	10.372
Total: Apr.-Sept.	3.832	3.951	3.662
Mean drainage per month	1.19108	1.23408	1.16950

the mid-point of the period 1878–1932. The values of c (constant in the regression equation (2)) for the different months and for the three gauges are given in Table XIII.

Comparing the values in Table III with those in Table I we find that, in all the gauges, the values of Table III are numerically lower from July to December and higher from January to June, with the exception of May; the apparent reason is that the standard rainfall used is higher than the actual (about 2 in.) for these months (January to June). It is a very remarkable fact that the seasonal effect remains practically the same after standardisation as before—the drainage values in the summer months being much lower than in the winter months. This is also clear from the totals of six-monthly periods—October to March, April to September—the value for the former being about three times the latter. If the behaviour of the drain gauge could be expected to depend only on the meteorology of the current month, these values should not have differed very much from month to month. The striking disparity with the expectation shows that much of the variation in drainage in the course of the year must be ascribed to the influence of the weather belonging not to the month of drainage but to a previous period. Comparing the three gauges, we find that, like the mean monthly drainage, the drainage under standard conditions is greater for the 40 in. gauge than for the 20 or 60 in. gauge.

Table IV. *Values of c' .*

Month	20 in. gauge P_1	40 in. gauge P_2	60 in. gauge P_3
Jan.	1.9043	2.0677	2.0073
Feb.	1.5622	1.6708	1.6086
Mar.	1.0936	1.2188	1.1625
Apr.	0.6807	0.7641	0.7302
May	0.5523	0.6280	0.5929
June	0.5366	0.5640	0.5502
July	0.7136	0.7406	0.7001
Aug.	0.7985	0.8170	0.7727
Sept.	0.7993	0.7963	0.7443
Oct.	1.7905	1.7829	1.6800
Nov.	2.1942	2.2575	2.1564
Dec.	2.4479	2.5528	2.4682
Total	15.0737	15.8605	15.1734
Mean	1.25614	1.32170	1.26444

The values of the three constants c' , b_2' and b_4' of equation (3) obtained for each month, for the three gauges, together with the values of b_1' , b_3' and b_5' which represent the steady change in these constants with time are tabulated in Tables IV–IX. We shall now discuss the

122 *Effects of Rainfall and Temperature on Drainage*

significance of these constants, and the anomalous behaviour of some of them.

Table V. b_1' . *Regression of drainage on t' .*

Month	20 in. gauge P_1	40 in. gauge P_2	60 in. gauge P_3
Jan.	+0.00214	+0.00136	+0.00053
Feb.	+0.00266	+0.00143	+0.00290
Mar.	+0.00146	-0.00104	-0.00058
Apr.	+0.00228	+0.00380	+0.00453
May	-0.00245	-0.00196	-0.00042
June	+0.00001	+0.00060	+0.00108
July	+0.00123	+0.00246	+0.00271
Aug.	+0.00388	+0.00594	+0.00636
Sept.	+0.00282	+0.00411	+0.00469
Oct.	-0.00042	+0.00132	+0.00205
Nov.	+0.00070	+0.00087	+0.00155
Dec.	+0.00359	+0.00360	+0.00405
Total	+0.01790	+0.02249	+0.02945
Mean	+0.00149	+0.00187	+0.00245

Table IV showing the values of c' , the expectation for the mean rainfall and temperature of each month, is in many respects similar to Table I, and indicates clearly the seasonal effect and the abnormal behaviour of the 40 in. gauge. Considering these values in conjunction with the values of b_1' (Table V) representing their steady rate of change, the following interesting points may be noted:

(i) The differences in the values of c' for the 40 in. gauge and the 20 in. gauge for January, February and March are likely to become smaller in later years in view of the fact that the values of b_1' , though small, are numerically less for the former than for the latter, particularly the value for the month of March for the 40 in. gauge is negative.

(ii) From April to October the difference $P_2 - P_1$ for c' is expected to become larger with advance of date in view of the larger value of b_1' for the 40 in. gauge.

(iii) The values of b_1' from April to December gradually increase from the 20 to the 60 in. gauge, consequently the differences in the values of c' for the three gauges would be less marked in later years.

(iv) As the numerical values of the differences in the values of b_1' for the different gauges are all very small the final values of c' for the year 1932 will not be much different from their values for the central date 1905.

The partial regression coefficients b_2' (regression on rainfall) are given in Table VI and their steady rate of change in Table VII. From the study of these tables the following very interesting points emerge.

(i) For all gauges the regression coefficients are higher in the winter

Table VI. b_2' . Regression of drainage on rainfall (R).

Month	P_1	P_2	P_3	$P_1 - P_2$	$P_2 - P_3$	$P_1 - P_3$
Jan.	0.96729 \pm 0.03848	1.01274 \pm 0.06123	0.98164 \pm 0.05290	- 0.04545 \pm 0.03758	+ 0.03110 \pm 0.02600	- 0.01435 \pm 0.03720
Feb.	1.03965 \pm 0.03693	1.04871 \pm 0.03940	1.02950 \pm 0.03659	- 0.00876 \pm 0.02456	+ 0.01921 \pm 0.01238	+ 0.01045 \pm 0.01393
Mar.	0.92797 \pm 0.05611	0.97816 \pm 0.06529	0.94363 \pm 0.06123	- 0.05019 \pm 0.01668	+ 0.03453 \pm 0.00979	- 0.01566 \pm 0.01688
Apr.	0.67706 \pm 0.04956	0.68270 \pm 0.05495	0.64398 \pm 0.05457	- 0.00564 \pm 0.01245	+ 0.03872 \pm 0.01638	+ 0.03308 \pm 0.01763
May	0.61128 \pm 0.04133	0.63423 \pm 0.04309	0.58589 \pm 0.04084	- 0.02295 \pm 0.01010	+ 0.04834 \pm 0.00973	+ 0.02539 \pm 0.01044
June	0.54450 \pm 0.05800	0.54770 \pm 0.05660	0.54177 \pm 0.05756	- 0.00320 \pm 0.00961	+ 0.00593 \pm 0.00486	+ 0.00273 \pm 0.00853
July	0.49579 \pm 0.04012	0.49820 \pm 0.04395	0.46855 \pm 0.04361	- 0.00241 \pm 0.01002	+ 0.02965 \pm 0.00529	+ 0.02724 \pm 0.00939
Aug.	0.69363 \pm 0.04891	0.69169 \pm 0.05060	0.68294 \pm 0.04855	+ 0.00194 \pm 0.00982	+ 0.02875 \pm 0.00482	+ 0.03069 \pm 0.00796
Sept.	0.72937 \pm 0.03738	0.73798 \pm 0.04301	0.71706 \pm 0.04384	- 0.00861 \pm 0.00792	+ 0.02092 \pm 0.00418	+ 0.01231 \pm 0.01006
Oct.	0.87745 \pm 0.03092	0.88269 \pm 0.03422	0.84146 \pm 0.03708	- 0.00524 \pm 0.01142	+ 0.04123 \pm 0.00892	+ 0.03599 \pm 0.01273
Nov.	0.97128 \pm 0.02757	1.01513 \pm 0.03085	0.97431 \pm 0.03225	- 0.04385 \pm 0.01496	+ 0.04082 \pm 0.01105	- 0.00303 \pm 0.01602
Dec.	0.98500 \pm 0.02676	0.96900 \pm 0.02590	0.93408 \pm 0.02733	+ 0.01600 \pm 0.01200	+ 0.03492 \pm 0.00922	+ 0.05092 \pm 0.01404
Mean	0.79338	0.80824	0.77707			

Table VII. b_3 . Change of regression on rainfall with time, or regression of drainage on R_t (rainfall sequence).

Month	P_1	P_2	P_3
Jan.	- 0.00042 \pm 0.00270	+ 0.00203 \pm 0.00429	- 0.00210 \pm 0.00370
Feb.	- 0.00223 \pm 0.00245	- 0.00321 \pm 0.00261	- 0.00213 \pm 0.00242
Mar.	- 0.00211 \pm 0.00433	- 0.00217 \pm 0.00504	- 0.00450 \pm 0.00473
Apr.	+ 0.00186 \pm 0.00314	+ 0.00035 \pm 0.00339	+ 0.00095 \pm 0.00337
May	+ 0.00403 \pm 0.00221	+ 0.00224 \pm 0.00230	- 0.00401 \pm 0.00218
June	+ 0.00253 \pm 0.00361	+ 0.00305 \pm 0.00353	- 0.00263 \pm 0.00359
July	+ 0.00314 \pm 0.00262	+ 0.00318 \pm 0.00288	+ 0.00348 \pm 0.00285
Aug.	+ 0.00047 \pm 0.00259	+ 0.00145 \pm 0.00268	+ 0.00198 \pm 0.00257
Sept.	- 0.00247 \pm 0.00232	- 0.00219 \pm 0.00267	- 0.00208 \pm 0.00273
Oct.	- 0.00488 \pm 0.00172	- 0.00476 \pm 0.00193	- 0.00419 \pm 0.00215
Nov.	+ 0.00053 \pm 0.00164	+ 0.00005 \pm 0.00183	+ 0.00065 \pm 0.00191
Dec.	+ 0.00391 \pm 0.00174	+ 0.00427 \pm 0.00168	+ 0.00498 \pm 0.00178
Total: Oct.-Mar.	- 0.00520	- 0.00579	- 0.00729
Total: Apr.-Sept.	+ 0.00450	+ 0.00198	+ 0.00580
Mean	- 0.000058	- 0.000317	- 0.000124

124 *Effects of Rainfall and Temperature on Drainage*

than in the summer months; particularly the value in July is about half that of February, showing that there is greater response to rainfall in the winter than in the summer months. It is interesting that the value for February is greater than unity, but in view of the large negative value of b_3 , the value falls off in succeeding years—thus for 1930 the values for the 20, 40 and 60 in. gauges are 0.98425, 0.96846 and 0.97800 respectively.

(ii) Considering the three gauges separately we find that P_2 is greater than either P_1 or P_3 (with the exception of August and December) indicating that in the 40 in. gauge there is, on the average, greater amount of drainage for an extra inch of rain than in the two neighbouring gauges. Had the 60 or the 20 in. gauge behaved exceptionally, we would have attributed the cause to the difference in the depths of the gauges, but as the 40 in. gauge has shown the abnormal effect, we suspect that the difference, like that in the average drainage, must be due to soil heterogeneity.

(iii) It is remarkable that the three gauges have behaved in a similar manner in response to rainfall, in spite of the great difference in their depth. Nevertheless, it is interesting to note that many of the small differences in the regression coefficients are significant as can be seen from the tabulated values of $P_1 - P_2$, $P_2 - P_3$, $P_1 - P_3$ with their associated standard errors. These small differences would not be considered significant in view of the large standard errors associated with each of the regression coefficients, but due to the fact that the residuals from the regression equations are highly correlated, the standard errors associated with $P_1 - P_2$, $P_2 - P_3$ or $P_1 - P_3$ are comparatively much smaller than would be expected if the residuals were independent. The significant differences $P_2 - P_3$ are shown by a cross-hatched portion of the histograms in Fig. 4, which shows diagrammatically these coefficients for the different months. From the figure it is clear that in nine months out of twelve this difference is significant, and even in the case of the three months (January, February and June), where it is non-significant, the difference $P_2 - P_3$ as in other months is positive, thus showing in an undoubted manner that the percolatability of the 40 in. gauge is decidedly higher than that of the 60 in. gauge. As the question of the significance of the difference of regression coefficients is of considerable importance and of wider application, it would be worth while if we consider in detail one or two months. The values of b_2' for the three gauges for the month of November are:

20 in. gauge	40 in. gauge	60 in. gauge
0.97128 \pm 0.02757	1.01513 \pm 0.03085	0.97431 \pm 0.03225

Judging from their standard errors, their values do not differ significantly from unity, consequently the difference between any pair of them might at first thought not be considered significant. But actually the small difference $+0.04082$ between the 40 and 60 in. gauge values is about four times its standard error (± 0.01105). The reason for this is that the residual covariance is nearly as great as the residual variance for any one of them, or in other words, the residuals obtained after fitting the regression formula are highly and positively correlated. The values of

REGRESSION OF DRAINAGE ON RAINFALL

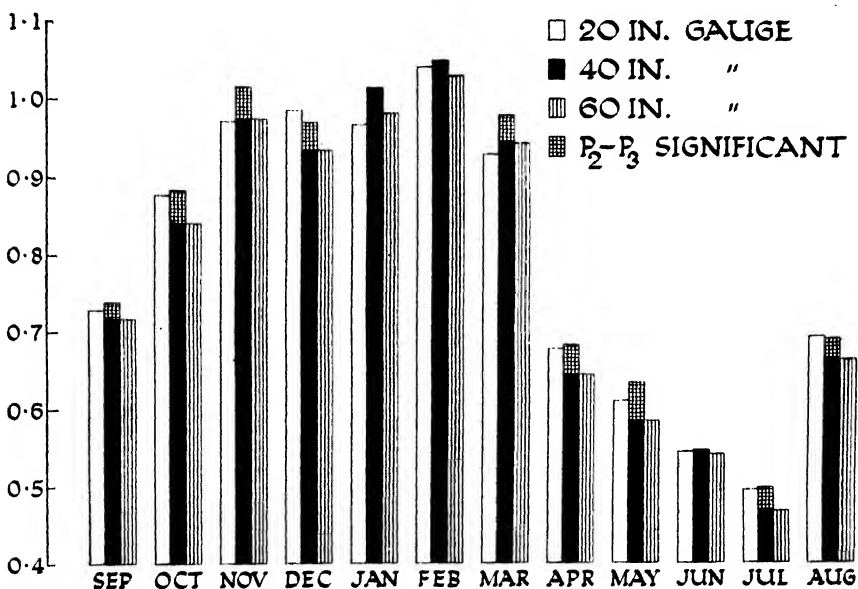


Fig. 4.

the correlations between the 40 and 60 in. gauges before and after elimination of regression are $+0.9969$ and $+0.8723$ respectively. In a similar manner the small difference $P_2 - P_3 = +0.02092$ for September is about five times its standard error (± 0.00418), for in this case the correlation between the residuals is even greater, the actual value being $+0.9721$.

The drain gauges may be compared to the neighbouring agricultural fields, and the example shows that conclusions with respect to the effect of weather on the yields of neighbouring plots similarly treated should be drawn with considerable caution. It may happen that the yields

126 *Effects of Rainfall and Temperature on Drainage*

after eliminating the effect of some element of weather, such as rainfall, may be highly correlated, and consequently the small difference between the regression coefficients may be highly significant, although considered separately each one of their standard errors may be large.

(iv) The values of b_3 given in Table VII indicate the change of regression on rainfall with date. Thus the value -0.00042 indicates that the regression on drainage for the 20 in. gauge in the month of January decreases at the rate of 0.00042 every year—thus its value in 1885 and in 1925 becomes 0.96813 and 0.96645 respectively, the value for the central date 1905 being 0.96729 . All the values of b_3 are small and non-

CHANGE OF AVERAGE REGRESSION ON RAINFALL WITH DATE

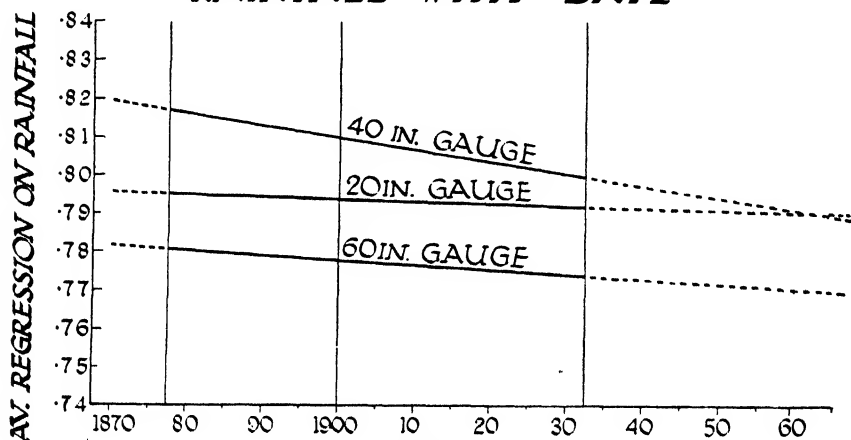


Fig. 5.

significant, except October and December, giving negative and positive significant values respectively. These small values of b_3 will not greatly affect the numerical values of the regression coefficients (b_2) themselves in succeeding years, but the difference between them for the different gauges would be materially altered in several cases. Thus $P_1 - P_2$ for December in the year 1930 would become $+0.00700$, which is about half its value $+0.001600$ for 1905. This is due to the fact that the value of b_3 ($+0.00391$) for P_1 which has given higher value of b_2' is lower than that ($+0.00427$) for P_2 giving lower value of b_2' , and if similar conditions hold good beyond 1932, the difference may disappear. For the same reason $P_1 - P_3$ would also become in 1930 about half its mean value (in 1905). The average values of b_3 given at the bottom of the table are negative for all the three gauges, and they represent the rate of change

of average values of b_2' given at the bottom of Table VI. This annual rate of change of average regression is illustrated graphically in Fig. 5. It shows that the difference between the gauges was considerably greater in the year 1878, but continuously became smaller in the succeeding years—particularly the difference between the 20 and the 40 in. gauges for the year 1932 is about one-quarter of that in 1878. If similar conditions continue it is expected that the difference may vanish in the near future.

REGRESSION OF DRAINAGE ON TEMPERATURE

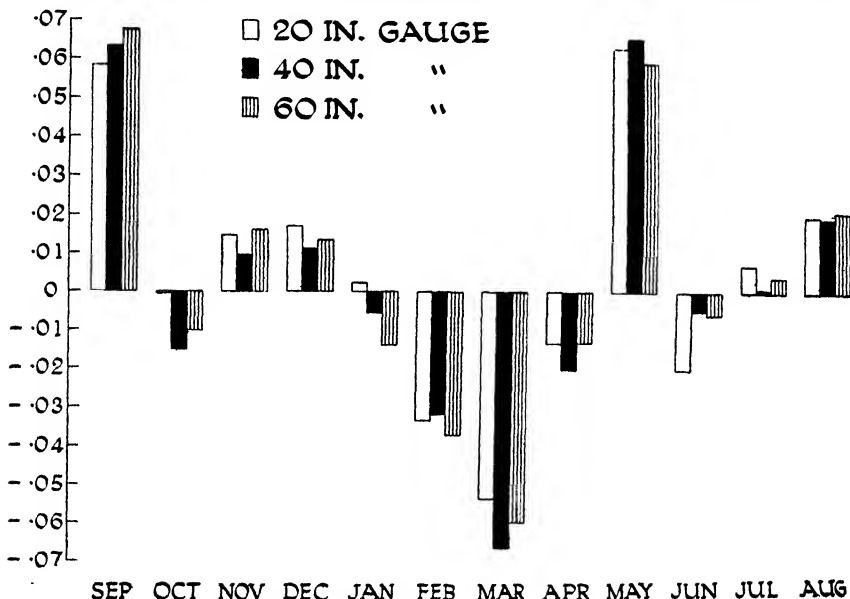


Fig. 6.

Table VIII gives the values of the regression of drainage on temperature and their steady change is indicated by values of b_5 given in Table IX. These have yielded very remarkable and in some cases paradoxical results. The general trend in the values for the three gauges can be seen from Fig. 6. Starting with a high positive value for September, we pass through a small negative value for October to small positive values for November, December and January (P_1 only), after which we get a succession of negative values, two of which for February and March are significant; after April there is a sudden return to a large positive and significant value for May, followed by a small negative value for June, and positive values for July and August.

Table VIII. b_4' . Regression of drainage on temperature.

Month	P_1	P_2	P_3	$P_1 - P_2$	$P_2 - P_3$	$P_1 - P_3$
Jan.	+0.00232 ± 0.01294	-0.00556 ± 0.02006	-0.01381 ± 0.01779	+0.00788 ± 0.01264	+0.00825 ± 0.00875	+0.01613 ± 0.01003
Feb.	-0.03361 ± 0.01361	-0.03194 ± 0.01452	-0.03717 ± 0.01348	-0.00167 ± 0.00905	+0.00523 ± 0.00456	+0.00356 ± 0.00513
Mar.	-0.05377 ± 0.02416	-0.06679 ± 0.02811	-0.06000 ± 0.02636	+0.01302 ± 0.00718	-0.00679 ± 0.00382	+0.00625 ± 0.00727
Apr.	-0.01339 ± 0.02647	-0.02022 ± 0.02945	-0.01314 ± 0.02914	+0.00683 ± 0.00665	-0.00708 ± 0.00875	-0.00025 ± 0.00942
May	+0.06332 ± 0.02277	+0.06591 ± 0.02375	+0.05948 ± 0.02250	-0.00259 ± 0.00557	-0.00643 ± 0.00436	+0.00384 ± 0.00510
June	-0.02037 ± 0.04190	-0.00483 ± 0.03993	-0.00593 ± 0.02250	-0.01544 ± 0.00678	+0.00100 ± 0.00343	-0.01444 ± 0.00602
July	+0.00712 ± 0.02440	+0.00080 ± 0.02674	-0.00395 ± 0.02653	+0.00632 ± 0.00610	-0.00315 ± 0.00322	-0.00317 ± 0.00566
Aug.	+0.01996 ± 0.03410	+0.01963 ± 0.03528	+0.02122 ± 0.03385	+0.00033 ± 0.00475	-0.00159 ± 0.00336	-0.00126 ± 0.00555
Sept.	+0.05831 ± 0.02630	+0.06332 ± 0.03015	+0.06762 ± 0.03073	+0.00501 ± 0.00555	-0.00430 ± 0.00286	+0.00931 ± 0.00706
Oct.	-0.00062 ± 0.01980	-0.01518 ± 0.02191	-0.01016 ± 0.02375	+0.01456 ± 0.00731	-0.00502 ± 0.00571	+0.00954 ± 0.00815
Nov.	+0.01469 ± 0.01366	+0.00962 ± 0.01528	-0.01517 ± 0.01597	+0.00507 ± 0.00741	-0.00550 ± 0.00572	-0.00048 ± 0.00802
Dec.	+0.01702 ± 0.01398	+0.01131 ± 0.01257	+0.01341 ± 0.01326	+0.00571 ± 0.00582	-0.00210 ± 0.00447	+0.00361 ± 0.00710
Total	+0.06098	+0.02597	+0.04064			
Mean	+0.00508	+0.00216	+0.00339			

Table IX. b_5 . Change of regression on temperature with time or regression of drainage on Tt (temperature sequence).

Month	P_1	P_2	P_3
Jan.	+0.00182 ± 0.00069	+0.00208 ± 0.00110	+0.00229 ± 0.00095
Feb.	+0.00033 ± 0.00093	+0.00052 ± 0.00099	+0.00038 ± 0.00092
Mar.	-0.00141 ± 0.00160	-0.00122 ± 0.00186	-0.00101 ± 0.00174
Apr.	-0.00085 ± 0.00187	-0.00024 ± 0.00202	-0.00012 ± 0.00200
May	+0.00002 ± 0.00145	-0.00037 ± 0.00151	+0.00026 ± 0.00143
June	+0.00125 ± 0.00296	-0.00016 ± 0.00289	+0.00011 ± 0.00294
July	+0.00238 ± 0.00179	+0.00200 ± 0.00196	+0.00192 ± 0.00195
Aug.	+0.00097 ± 0.00219	+0.00106 ± 0.00222	+0.00076 ± 0.00213
Sept.	-0.00251 ± 0.00145	-0.00284 ± 0.00167	-0.00294 ± 0.00171
Oct.	+0.00148 ± 0.00127	+0.00164 ± 0.00142	+0.00148 ± 0.00159
Nov.	+0.00011 ± 0.00089	+0.00069 ± 0.00099	+0.00078 ± 0.00104
Dec.	-0.00017 ± 0.00075	-0.00069 ± 0.00073	-0.00063 ± 0.00077
Total: Oct.-Mar.	+0.00216	+0.00302	+0.00329
Total: Apr.-Sept.	+0.00126	-0.00055	-0.00001
Mean	+0.003420	+0.002470	+0.003280

These regression coefficients show the response to temperature and indicate the average effect of an additional increment of 1° F. From reasoning *a priori* it is to be expected that an additional degree of temperature will cause greater evaporation and less drainage in all months; moreover, its effect would be greater in summer months than in winter, consequently we would have expected all the regression coefficients to be negative for all months and higher numerically in summer than in winter. But contrary to all expectations July and August, which are usually the hottest months in the year, instead of yielding high negative coefficients have given small *positive* values with the added feature that their rate of change as indicated by the values of b_5 are also positive, showing that their values in the succeeding years will tend to be positive and greater in magnitude. Thus the value $+0.01996$ for August for P_1 would become $+0.03936$ for 1925.

By far the most interesting and paradoxical result however is yielded by May where the value of b_2' is positive and highly significant for all the three gauges. The value for the 40 in. gauge is higher than the 20 or 60 in. gauges, but the small differences between them are not significant. It is interesting to note that the values of b_5 are positive for the 20 and 60 in. gauges, but negative for the 40 in. gauge, showing that small differences between the regression coefficients in 1905 will become small with the advance of date. However the negative value for P_2 (-0.00037) is small and does not change the sign of b_4 , its value for 1932 being $+0.05592$.

The second remarkable month, September, reveals a different story. Its value like that of May is large, positive and significant but due to the large negative and significant value of b_5 , it not only is falling off rapidly but actually changes its sign during the period of observation. Thus the high positive value $+0.05831$ for P_1 becomes a low negative value -0.00444 in 1930. For a similar reason, the small and negative values for P_2 and P_3 in January become large and positive values in 1930—the actual values being $+0.04744$ and $+0.04344$. In the month of March the three negative values -0.05377 , -0.06679 and -0.06000 become -0.08902 , -0.09729 and -0.08525 respectively in 1930. In this respect the regression on temperature differs from the regression on rainfall, where we found that due to the small values of b_3 (as compared with b_2) there was very slow change in the regression on rainfall with date.

The differences between regression coefficients for the different gauges P_1-P_2 , P_2-P_3 and P_1-P_3 are all small and non-significant except P_1-P_2 and P_1-P_3 for June, which is due to the comparatively high

130 *Effects of Rainfall and Temperature on Drainage*

though non-significant negative value -0.02037 for P_1 . It may be noted however that this negative value is associated with high positive value $+0.00125$ of b_5 , changing its value to $+0.01088$ for 1930. In general, if we thus consider the different values of b_4' in conjunction with their rate of change, we can divide them into two main classes—the first class consisting of three months, February, March and April, giving negative values for 1905 and 1930; and secondly the nine months, May to December and January, all of which (except September) give positive values with change of date.

CHANGE OF AVERAGE REGRESSION ON TEMPERATURE WITH DATE

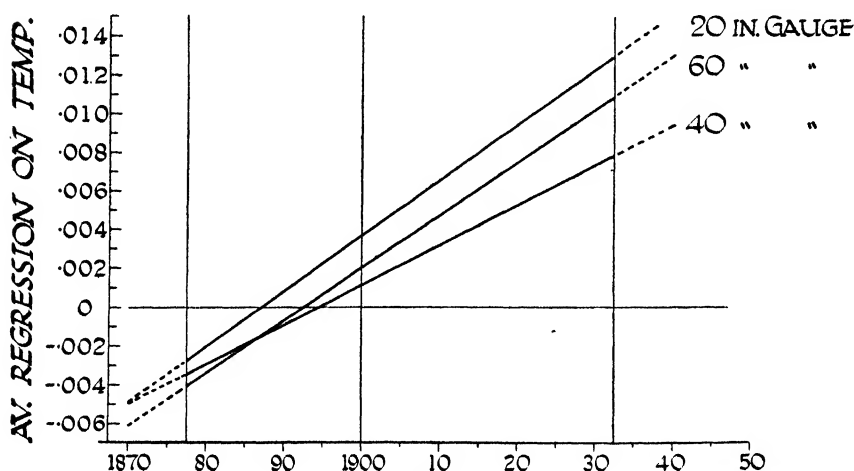


Fig. 7.

The average monthly regressions and their rates of change are all positive for all the three gauges—the actual value and its rate of change being least for the 40 in. gauge. Their trend at different dates can be seen from Fig. 7. They are all negative to begin with, but soon become positive, the difference between the 20 and 40 in. gauges being greater than in the other pairs.

Table X gives the values of the residual variance and shows how much of the variance in drainage for each month has not been removed by the regression formula for that month, and remains to be accounted for by other factors. Comparing these values with those of Table II, we find that unlike the variance, its values for November and December

are smaller than in other months, and this seasonal trend becomes more marked if we regard them as percentages of the original variance. Considering the variation between gauges we find that $P_2 > P_3 > P_1$ from January to August (except June), while for the three months, September, October and November, $P_3 > P_2 > P_1$.

Table X. *Residual variance s^2 .*

Month	20 in. gauge P_1	40 in. gauge P_2	60 in. gauge P_3
Jan.	0.074694	0.189041	0.141102
Feb.	0.118153	0.134388	0.115918
Mar.	0.187755	0.254143	0.223490
Apr.	0.129020	0.158633	0.156408
May	0.099673	0.108410	0.097367
June	0.229816	0.218980	0.226428
July	0.138735	0.166551	0.163898
Aug.	0.218551	0.233939	0.215367
Sept.	0.131775	0.174510	0.181347
Oct.	0.125429	0.153551	0.180592
Nov.	0.060857	0.076204	0.083245
Dec.	0.066102	0.061918	0.068939

The values of ψ^2 given in Table XI may be regarded as the values of residual variance of the difference between the pairs of gauges. Thus the values $\psi^2_{P_1-P_2}$ indicate the amount of variance not accounted for by the regression equation fitted to the differences P_1-P_2 . As would be expected, the values of $\psi^2_{P_2-P_3}$ are smaller than for the other two sets of pairs, on account of the comparatively higher correlation between the residuals of the 40 and 60 in. gauges. They also show a marked seasonal effect—the values being considerably smaller in summer.

Table XI. *Values of ψ^2 .*

Month	$\psi^2_{P_1-P_2} = (s_1^2 + s_2^2 - 2\rho_{12})$	$\psi^2_{P_2-P_3}$	$\psi^2_{P_1-P_3}$
Jan.	0.071205	0.034103	0.047592
Feb.	0.052255	0.013286	0.016805
Mar.	0.016592	0.004695	0.017001
Apr.	0.008143	0.014103	0.016326
May	0.005961	0.003491	0.005000
June	0.006306	0.001612	0.004978
July	0.008674	0.002409	0.007449
Aug.	0.004246	0.002122	0.005796
Sept.	0.005917	0.001571	0.009572
Oct.	0.017102	0.010429	0.021245
Nov.	0.017919	0.010673	0.021000
Dec.	0.013286	0.007837	0.019775

The values of residual percentage variance given in Table XII show a remarkable regularity. Their values are comparatively much smaller in October to February, showing that the regression formula has given greater precision in winter than in summer, and that the meteorological

Table XII. *Residual percentage variance.*

Month	20 in. gauge (P_1)		40 in. gauge (P_2)		60 in. gauge (P_3)	
	Observed	Harmonic curve	Observed	Harmonic curve	Observed	Harmonic curve
Jan.	7-1222	4-1283	15-2548	6-9752	12-6679	6-3179
Feb.	6-1799	8-7165	6-8515	11-5175	6-2109	10-9524
Mar.	14-6424	14-5984	17-6467	17-1163	16-9092	16-9649
Apr.	20-3394	20-1976	23-5800	22-2710	25-6569	22-7439
May	19-4863	24-0137	19-2418	25-6002	20-5423	26-7407
June	31-6333	25-0247	31-1198	26-2125	32-5284	27-8853
July	21-9094	22-9587	24-7482	23-9428	27-0541	25-8696
Aug.	18-3416	18-3705	19-7041	19-4005	19-9208	21-2352
Sept.	10-7553	12-4886	13-5444	13-8017	14-7416	15-2227
Oct.	5-8268	6-8894	7-0418	8-6470	8-9774	9-4437
Nov.	3-8860	3-0733	4-4485	5-3178	5-1815	5-4468
Dec.	2-3990	2-0623	2-3267	4-7056	2-7353	4-3023
Mean	13-5435	—	15-4590	—	16-0938	—

factors considered can account for the drainage in winter far more satisfactorily than in summer. In December about 98 per cent. of the drainage is accounted for by the regression formula, while in June only about 70 per cent. can be explained by the meteorology of the current month. These values have indicated a similar variability between the different gauges as is shown by residual variance values of Table X. These values show a genuine cyclic change, consequently harmonic curves of the form

$$P = \bar{P} + A \sin \theta + B \cos \theta,$$

where θ is zero at the mid-date of December, were fitted to these values and are shown graphically in Fig. 8. It shows that, in general, $P_1 > P_2 > P_3$, and the differences between the gauges are more marked in the central portions of the curves, which correspond to the summer months.

The values of \bar{P} , A , B and the analysis of variance for the three gauges are:

	P_1	P_2	P_3
\bar{P}	13-5435	15-4590	16-0938
A	1-05492	1-65733	0-87109
B	-11-48117	-10-75345	-11-79145
$A^2 + B^2$	132-93012	118-38343	139-79709

Analysis of variance (variance contributed by harmonic terms).

Variance due to	Degrees of freedom	P_1		P_2		P_3	
		Sum of squares	Mean square	Sum of squares	Mean square	Sum of squares	Mean square
Harmonic terms	2	797-58072	398-79	710-30058	355-15	838-7825	419-39
Deviation from harmonic curve	9	85-62331	9-51	166-65056	18-52	137-4025	15-27
Total	11	883-20403	80-29	876-95114	79-72	976-1850	88-74

The sum of squares removed by the harmonic terms are given by $6(A^2 + B^2)$.

It is extremely interesting to note that the major portion of the cyclic change is accounted for by the harmonic curve. Of the total sum of squares about 90 per cent. is accounted for by the two degrees of freedom due to the harmonic curve for the 20 in. gauge, 81 per cent. for the 40 in. gauge and 86 per cent. for the 60 in. gauge; leaving about 10 per cent.

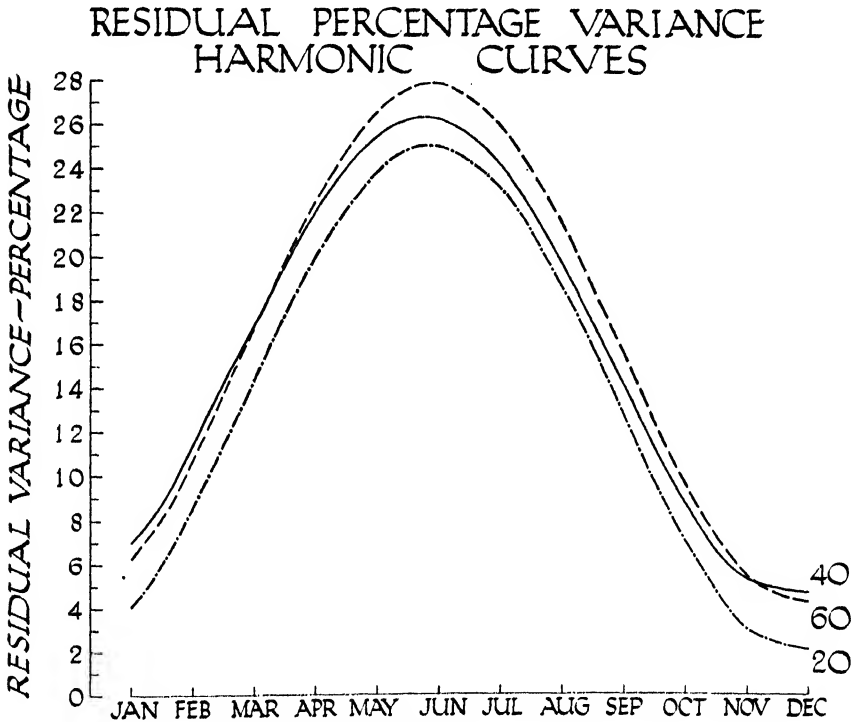


Fig. 8.

for the 20 in. gauge, 19 per cent. for the 40 in. gauge and 14 per cent. for the 60 in. gauge to be accounted for by other causes. It is remarkable to note that the percentage variance *not* accounted for by the harmonic curve is greatest for the 40 in. gauge, showing that it still behaves in an abnormal manner.

It is interesting to note that the values of \bar{P} indicating the mean percentage residual variance increase with the depth of the gauge, although no simple relation exists between these values and the depth.

134 *Effects of Rainfall and Temperature on Drainage*

Table XIII. *Values of c (c in the regression equation (2), i.e.*

$$P = c + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4 + b_5x_5).$$

Month	P_1	P_2	P_3
Jan.	-0.08338	0.35171	0.66822
Feb.	-0.28250	-0.20169	-0.18308
Mar.	-0.49607	-0.39416	-0.42209
Apr.	-0.64786	-0.54583	-0.53288
May	-0.81481	-0.81818	-0.72162
June	-0.61408	-0.64887	-0.64283
July	-0.55232	-0.51298	-0.49092
Aug.	-1.18496	-1.15416	-1.12978
Sept.	-1.31159	-1.37538	-1.40846
Oct.	-0.95291	-0.85117	-0.87034
Nov.	-0.66367	-0.67795	-0.69283
Dec.	-0.47471	-0.29257	-0.28521

SUMMARY.

1. Partial regression equations representing the average drainage observed in any month in terms of the temperature and rainfall of that month, and including terms representing the mean secular rate of change of the drainage discharge and of its regression coefficients on rainfall and temperature, have been fitted to the thirty-six series of observations provided by the three Rothamsted drain gauges in the twelve months of the year.

2. An account is given of adequate and direct numerical methods of handling equations involving observed quantities, and chosen functions of them, as independent variates, and of calculating standard errors appropriate to the several sorts of comparison which are to be made.

3. In the absence of direct knowledge of the amount of water contained from time to time in the soil mass of the gauge it has been customary to assume that the lower average drainage of the summer months is directly due to a greater amount of evaporation taking place in these months. The results of the present enquiry direct attention to a second possibility, namely that the water content of the gauges differs considerably at different times of the year, and that the high drainage in winter is in part to be ascribed to the accumulation of water during the rainy months of autumn, while the lower drainage in summer is due to the partial depletion of the gauges during the lower rainfall of the spring months. The statistical facts bearing on this point are as follows:

(i) When as in Table III allowance is made for the temperature difference between the different months of the year, the distribution of percolation is little altered on all three gauges.

(ii) The regressions of drainage on temperature are not uniformly or even predominantly negative, as would be the case if the only effect

upon drainage were due to an increase in evaporation. Table VIII shows on the contrary a prevalence in all three gauges of the positive coefficients.

(iii) The discharge from the 60 in. gauge is slightly but significantly delayed in the dates of its annual incidence compared to that of the 20 in. gauge, the 40 in. gauge being in this respect intermediate between the others. This difference can scarcely be ascribed to anything save the greater capacity of the soil in the deeper gauge.

4. The discharge from all three gauges has increased slightly during the period of observation, the increase being greatest in the 60 in., and least in the 20 in. gauge.

5. Both the average drainage and the average response to rain is greatest in the 40 in. gauge; with respect to the other two gauges the mean drainage is slightly higher for the 60 in. gauge, but the average response to rainfall is greater for the 20 in. gauge.

6. Very high and remarkable positive regressions of rainfall on temperature are observed for the months of May and September.

7. During the period of observation the regression of drainage on temperature has tended on the average towards increasing positive values.

8. The residual variations in drainage, not accounted for in terms of current rainfall and temperature, are in all months small compared to the actual values. In March, June and August, however, the standard error of prediction approaches half an inch. This residue could doubtless be further reduced by using a quadratic function of the monthly rainfall. Variation in previous weather, which, as has been shown, is evidently of great importance in comparing different months, can contribute relatively little to the variation among months of the same kind.

ACKNOWLEDGMENTS.

I am indebted to the Indian Central Cotton Committee for granting study leave and thus affording facilities to work at the Rothamsted Experimental Station.

REFERENCES.

- (1) FISHER, R. A. *Statistical Methods for Research Workers* (1925). Edinburgh: Oliver and Boyd, Ltd. Fourth edition, 1932.
- (2) RUSSELL, E. J. Note on an apparent secular change in the Rothamsted drain gauges. *J. Agric. Sci.* (1907) **2**, 29.

(Received October 10th, 1933.)

[*Reprinted from the Journal of Agricultural Science*, Vol. **XXIV**. Part I.]

[*All Rights reserved.*]

THE APPLICATION OF HEAT OF WETTING MEASUREMENTS TO SOIL RESEARCH PROBLEMS.

BY DR H. JANERT,

Privatdozent, Leipzig University, Germany.

*(Soil Physics Department, Rothamsted Experimental Station,
Harpenden, Herts.)*

(With Four Text-figures.)

I. INTRODUCTION.

THE heat of wetting measurement has been introduced into soil science by E. A. Mitscherlich⁽¹⁾, who constructed for his investigations a rather complicated apparatus, consisting of an ice calorimeter which had been adapted for the special requirements of soil tests. Mitscherlich used the heat of wetting as a measurement of the heaviness of different soils, but soon found the technique too difficult, and later preferred the well-known hygroscopicity determination which he had worked out in collaboration with H. Rodewald⁽²⁾.

The hygroscopicity and the heat of wetting figures show a close correlation, since both of them relate to the same process of liquid adsorption, although they do not measure the same thing. Since the heat of wetting had only been abandoned because of the intricacy of the method, it appeared very desirable to develop another technique, which should combine a sufficient accuracy with the necessary rapidity of determination. The author has taken up this problem and worked out a new method for the determination of the heat of wetting, which satisfies these requirements^(3, 4). An English description is given in a Technical Communication (No. 27, 1933) of the Imperial Bureau of Soil Science.

II. THE ACCURACY OF THE METHOD.

The method differs widely from the one which Mitscherlich used, and it is necessary to investigate whether the alterations are admissible or not.

First of all the drying is different, for Mitscherlich dried his samples over P_2O_5 in a vacuum at $100^{\circ}C$., but although this gives a more in-

tensive drying than an ordinary oven, the soil does not become absolutely dry. This is practically impossible, and it should be recognised that any method for drying soil samples is merely conventional. If the drying temperature is altered, the heat of wetting responds distinctly even to slight alterations, as shown in Table I.

Table I.

	Drying temperature (° C.)		
	105	110	155
	Heat of wetting in cal./gm.		
Soil 1	2.41	2.57	2.87
	2.39	2.50	
		2.57	
		2.53 Av. 2.54	
2	3.20	3.47	4.18
	3.22	3.43	
		3.47	
		3.43 Av. 3.45	
3	4.45	4.63	5.27
	4.43	4.63	
		4.60	
		4.64 Av. 4.62	

In these experiments three different temperatures were used, which were prescribed by the available equipment. The experiments give full evidence for the predominant importance of the drying temperature, and it is quite clear that satisfactory results can only be expected if the drying temperature is carefully kept constant.

Another problem is the duration of drying. It takes some time before an equilibrium is reached between the vapour pressure in the oven and of the soil, and therefore the time of drying must be long enough to be sufficient for all soils. In the experiments recorded in Table II an equilibrium appears to be obtained after 8 hours' drying at 110° C., but exceptionally heavy soils might require longer drying. The temperature

Table II.

	Drying temperature (° C.)							
	105		110				155	
Time of drying ...	16 hr.	36 hr.	4 hr.	8 hr.	16 hr.	40 hr.	23 min.	46 min.
Soil 1	2.41	2.44	—	—	—	—	2.87	2.78
	2.39	2.39						
2	3.20	3.26	3.42	3.46	3.47	3.48	4.18	4.14
	3.22	3.29	3.41	3.46	3.43	3.46		
3	4.45	4.40	4.40	4.59	4.60	4.59	5.27	5.25
	4.43	4.47	4.46	4.68	4.64	4.62		

and time of drying is therefore fixed at 110° C. and 16 hours, which is also a very convenient time, since the drying can then be carried out overnight.

The wetting process is also different in the new method. The soil certainly is more readily wetted if the wetting is done in a vacuum, but this entails considerable experimental difficulties. It is practically impossible to have both wetting under vacuum and thorough stirring. The stirring seems to be more important, since it not only serves to hasten the wetting process, but it also gives a quick equalisation of the temperature after the heat is evolved. This is of particular importance when, instead of an ice calorimeter, an ordinary calorimeter is used, since this is liable to experimental errors which increase with a prolongation of the determination.

III. HEAT EFFECTS RESULTING FROM LIQUID ADSORPTION BY THE SOIL.

The conceptions about the character and the origin of the heat effects measured in the heat of wetting have considerably changed lately. Formerly it was assumed that the soil surface adsorbs the water physically, covering itself with a certain number of molecular layers of water according to the vapour pressure. This conception was supported by measurements of the specific weight, which increases with the heaviness of the soil. Such an increase is certainly due to liquid condensation, but the question is, whether the condensation is caused by polar or non-polar adsorption. If both of them are active in the process of wetting soil, the heat of wetting should be higher with increasing polarity of the liquid. Actually water as a distinctly polar liquid produces a high heat of wetting, whereas a non-polar liquid, for example carbon tetrachloride, gives a smaller one, amounting to only about one-third of the water heat of wetting, where mineral soils are concerned. (The humus content of the soil causes considerable complications which will be dealt with in another section.)

For both polar and non-polar adsorption the effect is similar. The higher the dehydration, the more heat is evolved when the soil is re-wetted. If the heat of wetting of samples with different degrees of dehydration is measured, ranging from full saturation to the highest possible dehydration, curves are obtained like those in Fig. 1. In these experiments the samples were first dried all alike, and then a certain quantity of water added by putting a small tube with the water upright into the weighing glass containing the dry sample. The glass was sealed

and allowed to stand for a few weeks until the water was evaporated from the tube and adsorbed by the soil or the starch respectively. Later the heat of wetting was determined in the ordinary manner, and the quantity of water added to the dry sample was taken into account.

The curves for the water heat of wetting in Fig. 1 are very similar to those formerly obtained by E. A. Mitscherlich (5). They are quite regular

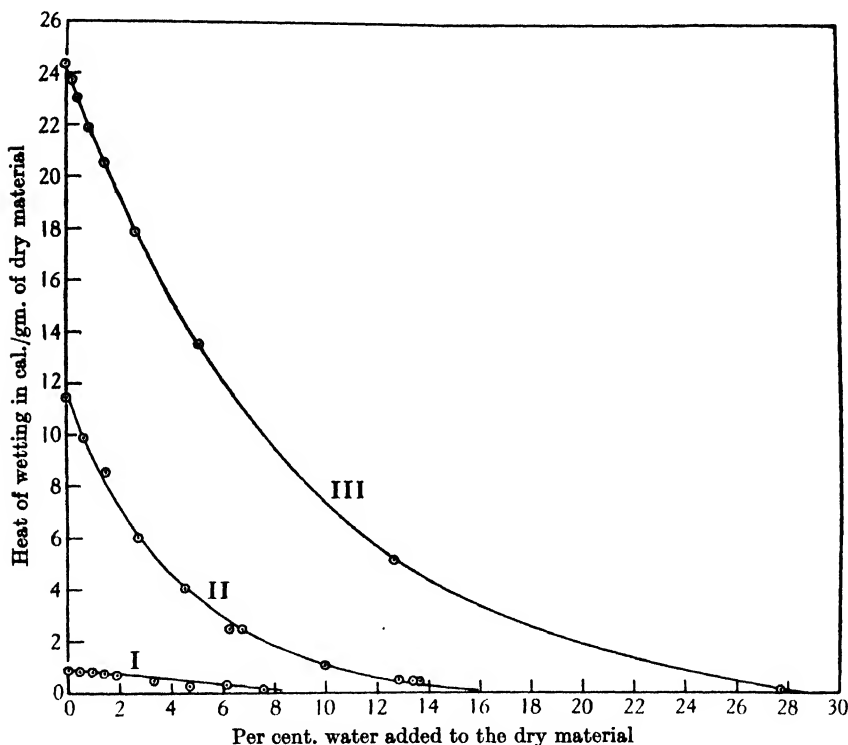


Fig. 1.

- I. Brick-clay wetted with carbon tetrachloride. II. Clay wetted with water.
III. Starch wetted with water.

and do not show any break or discontinuity. The distinctly different appearance of the carbon tetrachloride curve points to fundamental differences between the polar and non-polar adsorption, which will be shown more clearly in the experiments described below.

The polar adsorption should be closely correlated with the exchangeable bases held by the soil, and consequently there should be some correlation between the heat of hydration of the adsorbed cations and the heat of wetting obtained with a polar liquid. P. Vageler and

F. Alten (6) quote the heat of hydration of the main soil cations as follows:

	H	Na	K	Mg/2	Ca/2
Heat of hydration per milli-equivalent: cation (cal.)	247	94	75	229.5	174.5

These rather theoretical figures are valid for absolutely free ions which are quite inconceivable. The adsorbed cations are not free, but are bound to the alumino-silicate complex. Hence only a certain proportion of the total heat of hydration can reappear, when the dry soil is wetted, and this gives the heat effect that is called the heat of wetting. This proportion, however, must be approximately constant for any soil, if this theory of the origin of the heat of wetting is correct.

Thanks to the kind permission of Mr E. W. Russell to use some of his separated clays, I was able to prove the validity of this theory on a large scale with well-prepared material. The results of these experiments are given in Tables III and IV. The single-base clays had been obtained from hydrogen clay by a prolonged treatment with the acetates and subsequent washing with water or alcohol to remove the acetic acid and salts. Table III shows, besides the heat of wetting, the base-exchange capacity, determined by R. K. Schofield with his new phosphate method (7). The heat of wetting figures obviously follow the base-exchange capacity, and there is a close correlation between the total heat of hydration and the heat of wetting. The relation between these two values can be expressed by the quotient $\frac{\text{heat of hydration}}{\text{heat of wetting}}$, as shown in Table IV. This quotient is constant for all soils, the small variations being due to experimental errors and probably the impurity of the material. This indicates that the heat of wetting must be regarded as a portion of the total heat of hydration of the adsorbed cations. This proportion, however, differs considerably for the various cations, according to the intensity of their adsorptive binding. The adsorption is strongest for hydrogen, and therefore only 1/11.5 of the total heat of hydration is returned in the heat of wetting. Sodium, on the other hand, is but little adsorbed, and consequently a much higher proportion of the heat of hydration, viz. 1/4.9, appears in the heat of wetting. The other cations fall between and follow each other in the well-known order of intensity of adsorptions:

H	>	Mg/2	>	Ca/2	>	K	>	Na
1/11.5		1/9.0		1/7.0		1/5.1		1/4.9

These results are confirmed by the measurements of L. D. Baver (8),

Table III.

Separated clay sample	Base-exchange capacity milliequivalent 100 gm. (R. K. Schofield)	H		Mg/2		Ca/2		K		Na	
		Heat of		Heat of		Heat of		Heat of		Heat of	
		Wetting	Hydration	Wetting	Hydration	Wetting	Hydration	Wetting	Hydration	Wetting	Hydration
		cal./gm.	cal./gm.	cal./gm.	cal./gm.	cal./gm.	cal./gm.	cal./gm.	cal./gm.	cal./gm.	cal./gm.
Triassic clay	25	5.36	61.75	6.28	57.38	6.49	43.62	3.76	18.75	4.84	23.50
Barnfield	26	5.44	64.22	—	—	6.08	45.37	3.78	19.50	4.94	24.44
Carse	40	8.61	98.80	—	—	9.96	69.80	—	—	—	—
Rothamsted	41	8.96	101.27	—	—	10.46	71.54	5.80	30.75	7.51	38.54
Lower Lias	42	9.05	103.74	10.71	96.39	10.41	73.29	6.27	31.50	8.08	39.48
Oolite	45	—	—	—	—	11.37	78.52	—	—	9.06	42.30

Table IV.

Separated clay sample	Heat of hydration			
	Heat of wetting			
	H	Mg/2	Ca/2	Na
Triassic clay	11.5	9.1	6.7	5.0
Barnfield	11.8	—	7.5	5.2
Carse	11.5	—	7.0	4.9
Rothamsted	11.3	—	6.8	—
Lower Lias	11.5	9.0	7.0	5.3
Oolite	—	—	7.0	5.0
Average	11.5	9.0	7.0	5.1
				4.9

who determined the heat of wetting for several pure single-base clays. Since the base-exchange capacity of these clays is not given in his paper, it has been calculated (Table V) from the figures obtained for the calcium clays, which are probably the most accurate ones. A direct comparison is then possible between the heat of wetting figures determined by L. D. Bayer and those calculated from the heat of hydration by using the factors given above.

Table V.

Soil	Base-exchange capacity calculated	Treatment (cation)	Heat of wetting	
			Determined (Bayer) cal./gm.	Calculated (Janert) cal./gm.
Toledo silty clay, 0-5 in.	30.1	H	6.50	6.47
		Mg	7.61	7.67
		Ca	7.53	7.53
		K	3.98	4.42
		Na	5.99	5.78
Toledo silty clay, 14-18 in.	25.4	H	5.86	5.46
		Mg	6.99	6.48
		Ca	6.34	6.34
		K	3.74	3.73
		Na	4.31	4.88
Ellsworth silt loam, 17-28 in.	13.6	H	3.63	2.92
		Mg	3.88	3.47
		Ca	3.40	3.40
		K	2.08	2.00
		Na	2.96	2.61
Ellsworth silt loam, 28-30 in.	14.1	H	3.28	3.03
		Mg	3.53	3.59
		Ca	3.53	3.53
		K	2.00	2.07
		Na	2.25	2.71
Clermont silt loam, 0-8 in.	10.2	H	2.44	2.19
		Mg	2.64	2.60
		Ca	2.56	2.56
		K	1.76	1.50
		Na	1.92	1.96

Considering the experimental difficulties and the fact that Bayer and the present writer used different methods for their determinations, the agreement is very satisfactory (Table V).

It would be expected that the close correlation between the base-exchange capacity and the heat of wetting, which has been observed for the rather artificial single-base clays, exists also for natural soils. The large differences in the heat of wetting shown by the prepared clays are not likely to appear in natural soils, because there are no pure single-base soils. Generally all the main soil cations are represented in the adsorbing complex, though not in the same proportion, since one or two are usually prevalent and others deficient. This causes alterations

in the hydration forces and consequently in the heat of wetting, which can therefore not show a linear correlation with the base-exchange capacity, but only a general correspondence. This is entirely confirmed by experiments carried out with a large variety of soil samples, including salt soils from India, lateritic soils from the Gold Coast and others from South Africa, the Sudan and Palestine, in addition to about a dozen

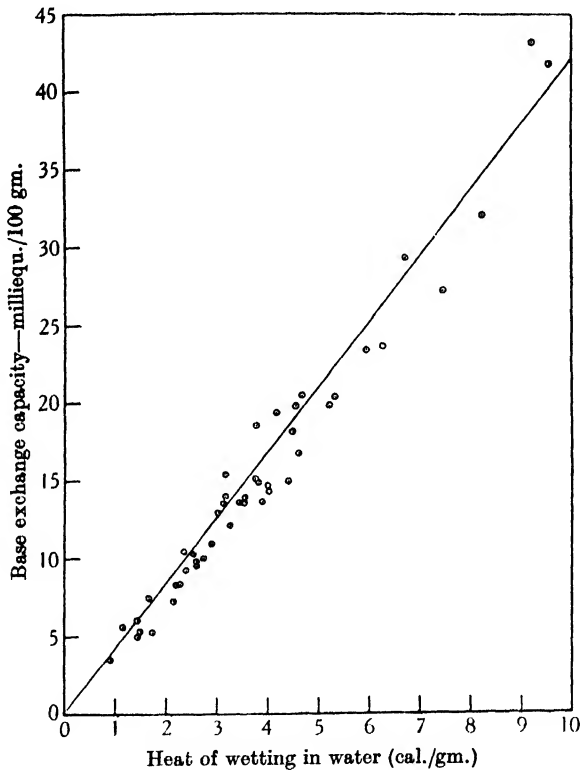


Fig. 2.

samples from various parts of England. I am very much indebted to Dr R. K. Schofield who did the phosphate tests, the results of which are given in Fig. 2, in relation to the heat of wetting.

Among the adsorbed cations there is generally little sodium and potassium, their adsorption being very weak. If we therefore suppose the remaining cations to be adsorbed on the average in equal amounts, the values for the base-exchange capacity should be theoretically about 4.2 times the heat of wetting figures, as indicated by the straight line

in Fig. 2. The results group themselves quite satisfactorily along this line, thus confirming the theory developed above.

Interesting correlations can also be established between the heat of wetting of a soil and its "heaviness" under certain conditions. Since the heaviness of a soil is a very complex conception which has not yet been specifically defined, it cannot be directly measured by any physical method. One of the most direct measurements of the heaviness of a soil is the draw-bar pull exerted by a tractor when ploughing. The heat of wetting of a series of samples taken in the course of measuring the draw-bar pull required to plough Broadbalk Field at Rothamsted was determined, and the results are given in Fig. 3. Although the field

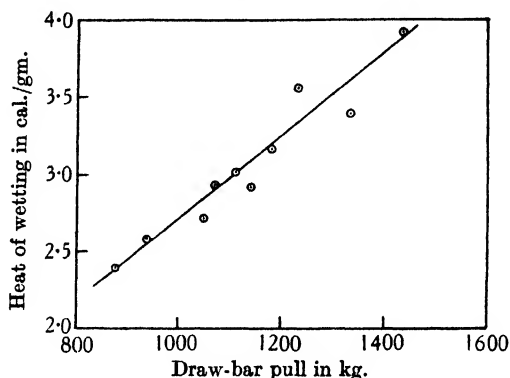


Fig. 3.

measurements and laboratory tests appear to be entirely different, there is quite a good correlation between these two quantities. This is not so surprising, since W. B. Haines and B. A. Keen(9) showed the draw-bar pull was closely correlated with the clay content. These authors found that the draw-bar pull and the ease of drainage were closely correlated, and this is borne out by drainage investigations in Germany(10), which have also shown that the heat of wetting gives a good indication of the ease with which a soil will drain under field conditions.

IV. THE WETTING WITH ORGANIC LIQUIDS.

The attempt has already been made to use organic liquids for soil physical tests. E. A. Mitscherlich(5) has used in his wetting experiments various organic liquids in comparison with water, and he has found that the heat effects follow in principle the same rules, although they show considerable differences in magnitude. A remarkable exception was,

however, encountered when humus soils or starch were tested. These materials gave a very high heat of wetting in water, but only a small heat effect in organic liquids. Mitscherlich concluded from his results that these materials are characterised by a microporous structure, thus developing a so-called "inner" surface, which is easily wetted with water but not with organic liquids, the molecules of which are too big to pass the narrow pores. He proposed to distinguish between an "outer" and an "inner" surface, assuming that the latter can only be wetted by water.

This theory disregards the influence of the polarity of the different liquids, which should be considerable. A detailed study of this question appears very desirable, since it might lead to a better knowledge of the adsorption processes in the soil. The author took up this question and carried out a set of experiments, which were done on soils practically free from humus, in order to avoid disturbances due to organic material. Six samples, as different as possible, were selected, and the heat of wetting determined in water and four organic liquids. The results are given in Fig. 3, where the heat of wetting obtained with the organic liquids is plotted against the water heat of wetting. The non-polar carbon tetrachloride produces the smallest heat effect. The values for paraffin, though also non-polar, lie a little higher, which is probably due to some impurity. The polar liquids, xylene and nitrobenzene, give a much higher heat of wetting, nearing that obtained with water. The latter is, however, still higher, although the dipole moment of water is only $1.8 \cdot 10^{-18}$, *i.e.* considerably smaller than the dipole moment of nitrobenzene which is $3.9 \cdot 10^{-18}$. This seems to show that the polarity is not the only criterion determining the adsorptive heat effects, but the volume of the molecule plays also a part according to the older conception of Mitscherlich.

Even these two factors do not suffice to explain the comparatively high values for xylene. These should be much smaller than the nitrobenzene figures and only slightly higher than the carbon tetrachloride heat effects. So far no explanation can be given for this discrepancy.

The behaviour of the humus soils which were tested besides the mineral soils given in Fig. 4 was very interesting. The results for the mineral soils fall in a straight line, but any soil containing humus was clearly out of that line, the deviation being obviously determined by the humus content.

If the quotient $\frac{\text{water heat of wetting}}{\text{organic liquid heat of wetting}}$ is constant for mineral

soils, regularly increasing with progressive humus content, as has been qualitatively observed, it might be possible to work out a rapid method for a quantitative determination of the humus content. This is now being investigated in collaboration with J. L. Russell.

As a preliminary study some Russian soil profiles have been tested and for the different horizons the "humus quotient" was determined, using carbon tetrachloride as organic liquid. These experiments give a rough idea of how the humus quotient may be used in soil science. Its

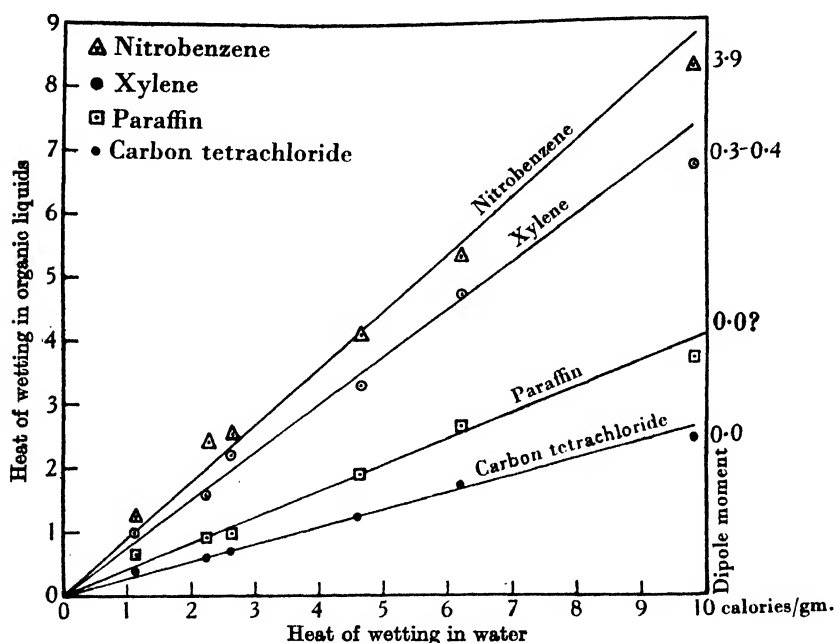


Fig. 4.

value would of course be much greater if it actually were possible directly to calculate the humus content from it.

The experimental data given in Table VI show the humus quotients for two very different soil types. The highest humus quotient is found in the chernozem, horizon A, decreasing steadily down to horizon C, thus indicating a deep and fertile soil. In the podsol profile the different horizons are most clearly differentiated. The accumulation of humus is restricted to the surface layer, decreasing rapidly from A_1 to A_2 . Horizon B_1 is extremely poor, deprived of colloidal material and distinctly less fertile than the underlying subsoil in B_2 and C.

Table VI. *Russian profiles.*

Soil type	Horizon	Identification	Heat of wetting cal./gm.		Humus quotient
			H ₂ O	CCl ₄	
Podsol (Detzkoye Selo)	A ₁	A. 1297	3.21	0.38	8.45
	A ₂	A. 1298	1.76	0.37	4.75
	B ₁	A. 1299	1.27	0.40	3.18
	B ₂	A. 1300	2.30	0.58	3.96
	C	A. 1301	2.65	0.70	3.79
Chernozem (Izberdey)	A	A. 1314	8.29	0.65	12.75
	B	A. 1315	7.21	0.89	8.10
	C	A. 1316	5.32	1.14	4.67

V. AN APPLICATION OF THE METHODS DESCRIBED TO "CLASSICAL FIELDS" AT ROTHAMSTED AND WOBURN.

The classical fields at Rothamsted provide a unique opportunity for investigating the influence of a very prolonged manurial treatment on the physical conditions of the soil. In the case of Broadbalk Field the manuring repeated every year in exactly the same way over a period of nearly a century should distinctly demonstrate an alteration of the physical conditions, if such an alteration is at all caused by the treatment practised on the different plots. Samples were taken from those plots showing the greatest differences in treatment and yield, and these samples were tested by the methods described above. The results are given in Table VII, where the figures of columns 5 and 6 are most important.

The "degree of natural dispersion" quoted in column 5 is determined by a combination of the heat of wetting measurement with a simple elutriation test. The latter serves to determine the proportion of fine particles with a diameter of less than 0.02 mm., which is, however, not identical with the equivalent fraction of the mechanical analysis, since no artificial dispersion is applied in the elutriation. Therefore the percentage of fine particles as determined by elutriation is greatly influenced by the soil structure. For a medium soil the percentage of particles < 0.02 mm. is about eight times the heat of wetting expressed in cal./gm. This factor is smaller for soils showing a well-developed crumb structure, but it exceeds 10, and is sometimes even greater than 15 for soils in bad structure. It is therefore successfully used as a measure for the "degree of natural dispersion" of soils (11, 12).

The figures for Broadbalk Field (Table VII, column 5) lie round about 8, but there are differences which are just significant. Plot 2 shows by far the best structure, as was to be expected, since this plot

has regularly received farmyard manure. It therefore has also the highest humus quotient (column 6). The worst structure is found on plot 5 receiving minerals only. The other differences do not seem to be significant enough to draw conclusions from them.

Table VII. *Broadbalk Field.*

1 Plot No.	2 Treatment	3 Heat of wetting cal./gm.		4 Particles <0.02 mm. %	5 Degree of natural disper- sion	6 Humus quotient H_2O/CCl_4 , heat of wetting
		In water	In CCl_4			
2	Dung	3.70	0.62	17.7	4.8	6.0
3	Unmanured	2.50	0.73	20.2	8.1	3.4
5	Complete minerals	2.39	0.70	20.8	8.7	3.4
6	Minerals + 206 lb. N	2.52	0.68	20.6	8.2	3.7
7	Minerals + 412 lb. N	2.95	0.73	21.6	7.3	4.0
8	Minerals + 618 lb. N	2.96	0.66	18.2	6.1	4.5
9	Minerals + 275 lb. nitrate of soda	2.64	0.61	20.9	7.9	4.3

N means in this table "sulphate of ammonia."

The humus quotient (column 6) is only 3.4 on the unmanured plot and the same on No. 5 with complete minerals. These plots are both very poor in humus, but the humus content goes up with increasing nitrogen dressings (plots 6, 7, 8), as is clearly shown by the humus quotient.

On the whole, the differences observed on Broadbalk are very small, considering the permanency of the treatment. The heavy soil of Broadbalk resists very well the influences of the one-sided treatment, and the reserves of such a soil will probably prevent much greater changes in structure for some long time to come. The effect should be quite different on a light soil, and it is very fortunate that similar experiments could

Table VIII.

1 Plot No.	2 Treatment	3 Heat of wetting cal./gm.		4 Particles <0.02 mm. %	5 Degree of natural disper- sion	6 Humus quotient H_2O/CCl_4 , heat of wetting
		In water	In CCl_4			
31	Sulphate of ammonia alone	1.42	0.36	14.3	10.1	3.9
32	Sulphate of ammonia + minerals	1.47	0.40	13.4	9.1	3.7
33	Sulphate of ammonia + lime	1.56	0.39	8.0	5.1	4.0
34	No manure	1.37	0.36	6.8	5.0	3.8
35	Nitrate of soda	1.52	0.36	8.7	5.7	4.2

be repeated on the light Woburn soil, where some of the permanent barley plots provided the material for the laboratory tests, the results of which are shown in Table VIII.

Although the Woburn field experiments were laid out much later than those on Broadbalk Field, there is already a very marked effect of the manuring on the physical condition of the soil, particularly its structure. The soil structure is very good on the unmanured plot with a degree of natural dispersion of 5.1, but there is an enormous deterioration on the plots receiving sulphate of ammonia. Here the degree of natural dispersion is 9.1 and even 10.1, *i.e.* a doubling of the natural dispersion. The plot which receives lime in addition to sulphate of ammonia is, however, normal. This shows that it is only the calcium which is already exhausted by continuous physiologically acid manuring in this light soil. The nitrate of soda, on the other hand, has only produced a small change of the natural dispersion, which is in accordance with field observations.

The humus quotient shows practically no change on the few plots that were tested. Apparently there is little accumulation of humus in this light dry soil.

We may conclude from these experiments that certain changes of the physical conditions in the soil on the permanent experimental fields at Rothamsted and Woburn have been clearly distinguished by the new methods applied.

VI. CONCLUSIONS.

1. Experiments carried out with pure single-base clays have shown that the heat of wetting represents a specific proportion of the heat of hydration of the adsorbed cations in their free state.

2. Correlations exist between the heat of wetting and various other soil properties determined in the field or by laboratory methods.

3. Wetting with organic liquids produces a heat effect which is proportional to the water heat of wetting for mineral soils. The dipole moment and the molecular volume of a liquid do not seem to determine completely the heat of wetting of a given soil in it.

4. New methods were applied to some of the permanent plots at Rothamsted and Woburn, where changes in the physical condition of the soil could be distinguished.

ACKNOWLEDGMENT.

I wish to thank Dr R. K. Schofield and the other members of the Soil Physics Department at Rothamsted for the valuable assistance that they readily gave me throughout the course of the experimental work.

REFERENCES.

- (1) MITSCHERLICH, E. A. Dissertation, Kiel (1899).
- (2) RODEWALD, H. and MITSCHERLICH, E. A. *Landw. Versuchs-Stat.* (1903), p. 433.
- (3) JANERT, H. *Z. f. Pflanzenern., Düng. u. Bodenk.* (1931), A, 19, 281.
- (4) — *Landw. Jahrb.* (1927), 66, 425.
- (5) MITSCHERLICH, E. A. *Landw. Jahrb.* (1901), 30, 380.
- (6) VAGELER, P. and ALTEN, F. *Z. f. Pflanzenern., Düng. u. Bodenk.* (1931), A, 21, 335.
- (7) SCHOFIELD, R. K. *J. Agric. Sci.* (1933), 23, 255.
- (8) BAVER, L. D. *J. Amer. Soc. of Agronomy* (1928), 20, 921.
- (9) HAINES, W. B. and KEEN, B. A. *J. Agric. Sci.* (1925), 15, 394.
- (10) ZUNKER, F. *Der Kulturtechniker* (1928), 31, 38.
- (11) JANERT, H. *Transact. of the VI Comm. of the Intern. Soc. Soil Sci.* (1932), vol. A, 163.
- (12) — *Imp. Bureau of Soil Sci.* (1933), Tech. Commun. No. 27.

(Received October 18th, 1933.)

ON THE MEASUREMENT OF IMBIBITIONAL WATER.

By E. W. RUSSELL AND R. S. GUPTA.

(*Soil Physics Department, Rothamsted Experimental
Station, Harpenden, Herts.*)

RECENTLY one of the authors⁽¹⁾ criticised the theory of the determination of the water imbibed by the soil colloids proposed by Fisher⁽²⁾ on the ground that it contained several unproved assumptions, some of which are examined in the present paper. Fundamentally, the imbibitional water theory assumes that the water held by a soil can be divided into two parts, colloiddally bound or imbibed water, and free water, the latter being held in the soil pores and capillaries. The method proposed by Fisher for determining the weight of water imbibed by a soil assumes that it equals the difference between the volume of water and the volume of xylene or other non-polar hydrocarbon the soil can hold in a centrifugal field of 1000 g., these volumes being calculated from the weight of the water or xylene held. This method therefore contains the following assumptions:

(1) The soil colloids do not imbibe xylene or other non-polar hydrocarbons.

(2) The imbibition of water is complete under a centrifugal field of 1000 g.

(3) The density of the liquid in the fine pores is the same as the bulk density of the liquid.

(4) The volume of the hydrocarbon held in the soil pores in the centrifugal field equals the volume of water so held.

A priori, there is no reason why the last assumption should be true, for two different processes, which work in opposite directions, can invalidate it. A soil swells on imbibing water and this swelling will probably alter the pore-space geometry of the fine pores by reducing their volume. But the surface tension of the soil-water-air interface is probably much greater than that of the soil-hydrocarbon interface, so that under a given centrifugal field there will be some capillaries and pores too large to hold the hydrocarbon but small enough to hold water.

The method employed in this investigation was to determine the

weight of water and of different hydrocarbons held by the soil under two different external conditions, namely, in the moisture equivalent centrifuge against a field of 1000 g. and in the Keen-Raczkowski box. For this investigation the following four soils were used:

Barnfield Rothamsted surface soil plot 1-0, which has received 14 tons of farmyard manure every year since 1856, the sample being taken from a depth 0-6 in.

Barnfield Rothamsted subsoil from plot 8-0, which has received no manure since 1853, the sample being taken from a depth of 3-4 ft.

Gezira soil taken from the Wad Medani Experimental Station, Sudan.

Gloucester soil, from Andoversford, Gloucester, which has been formed from the weathering of an inferior oolitic limestone.

All the soil samples were taken several years ago, and have since been stored in the air-dry condition at Rothamsted. Before use they were ground to pass a 1 mm. sieve. The liquids used, with the exception of the paraffin, were obtained from the British Drug Houses, Ltd., and their description in the text corresponds with that in the B.D.H. catalogue. The paraffin used was ordinary lamp oil dried over lime. The liquids were not discarded after use, but were shaken up with lime, filtered and re-used. With the exception of the paraffin there is no evidence of any progressive change of properties, but the paraffin had to be put in contact with dry soil once before it gave steady values.

For the determination of the liquid equivalent of these soils a standard Briggs-McLane centrifuge fitted with a constant speed regulator was used. The method of working and the boxes used are the same as used by Coutts(3), the only differences being that oven-dry soils only were used, and that, since many of the liquids used were not volatile, the initial weight of soil put in the box and the weight of soil + liquid taken out of the box only were determined. Whenever a volatile liquid was used, this soil was then dried, and the weight of dry soil removed always agreed with the weight of the dry soil put in the box to within a few milligrams.

The boxes used for the Keen-Raczkowski experiment were 3 cm. in diameter and 1.6 cm. high. The filter paper was kept down by a bent piece of steel spring wire, as described by Coutts(4). The boxes were packed and over-filled with air-dry soil, put in a 110° C. oven for 18 hours, allowed to cool, gently repacked and the surplus soil scraped off. The boxes were then weighed and either put in the wetting liquid to a depth just covering the bottom of the box, or put in a desiccator to be wetted *in vacuo*. For the vacuum wetting the desiccator was evacuated very slowly on a Hyvac oil pump, to allow the air held by the soil to

escape without disturbing the packing. Sufficient liquid was then let in to allow the liquid surface to touch the bottom of the box, so that the adsorbed air which was displaced by the wetting liquid could escape slowly either through the filter paper or from the soil surface. The liquid level was then raised 3-4 mm. above the bottom of the box and the soil allowed at least 12 hours to come into equilibrium with the liquid. Dry air was then let into the desiccator, the box removed, weighed, and the soil that had swollen above the top of the box sliced off. This surplus soil was then washed free from the hydrocarbon with benzene, and dried.

The drying temperature at which the soils were oven-dried appeared to be unimportant, for no differences were found within experimental error between the weight of liquid held by a soil in either experiment when it was dried for 18 hours at 105 or 110 or 120° C. In all the experiments recorded here, the soils were dried for 18 hours at 110° C.

THE EXPERIMENTAL RESULTS.

The liquid equivalents of the four soils are given in Table I; they are expressed as the volume of liquid in c.cm. held by 100 gm. of oven-dry soil against a centrifugal field of 1000 g., the volume being obtained by dividing the weight of the liquid held by its bulk density.

Table I. *The liquid equivalents of the soils.*

(Volume of liquid held per 100 gm. of soil in the moisture equivalent centrifuge.)

	Barnfield surface	Barnfield subsoil	Gezira	Gloucester	Mean
Water	25.0	37.0	40.3	27.2	—
Paraffin	14.3	17.4	10.8	11.1	13.4
Tetralin	14.8	18.1	10.6	11.4	13.7
Tetralin (purified)	15.1	18.1	10.4	12.1	13.9
Decalin (purified)	14.6	17.0	9.9	11.2	13.2
Xylene (rectified)	14.0	15.8	9.9	10.1	12.4
Xylene (sulphur-free)	12.7	16.4	9.4	10.1	12.1
Hydrocarbon mean	14.2	17.1	10.2	11.0	

The hydrocarbon equivalents are much lower than the water equivalent, and only show small deviations from one another. The lowness of the xylene equivalent is probably due to some xylene evaporating during centrifuging, and the difference between the decalin and tetralin equivalents is probably outside experimental error. No explanation of this small difference can be given, though it is unlikely that it is due to impurities in the tetralin since the purified and commercial liquids give the same equivalents.

The results for the Keen-Raczkowski box experiment are given in Table II, where, as before, the volume of liquid in c.cm. held by 100 gm.

of oven-dry soil is tabulated, the volume being calculated from the weight of liquid held on the assumption that the density of the liquid in the soil is the same as its bulk density.

Table II. *Volume of liquid held per 100 gm. of oven-dry soil in the Keen-Raczkowski box.*

	Barnfield surface	Barnfield subsoil	Gezira	Gloucester	Mean
Water	42.1	59.1	81.7	59.5	—
Paraffin	37.3 (38.2)	37.5 (40.7)	48.3 (44.3)	48.7 (40.0)	42.7 (40.8)
Tetralin (purified)	38.5 (38.2)	39.1 (40.0)	52.5 (44.6)	47.1 (41.0)	44.3 (40.9)
Decalin (purified)	37.4	38.0	50.6	44.3	42.6
Xylene (rectified)	36.1 (38.6)	40.2 (40.3)	50.3 (42.0)	44.0 (40.6)	42.6 (40.4)
Carbon tetrachloride (A.R.)	36.0 (36.2)	36.7 (38.7)	47.5 (38.4)	44.7 (38.7)	41.2 (38.0)
Benzene (A.R.)	37.4 (38.6)	38.6 (40.7)	48.5 (47.0)	46.5 (42.9)	42.7 (42.8)
Hexane (B.P. 67–69°)	38.9 (38.3)	39.0 (41.3)	51.1 (46.2)	47.1 (43.8)	44.0 (42.4)
Hydrocarbon mean	37.4 (38.0)	38.4 (40.3)	49.8 (43.7)	46.1 (41.2)	—

The figures in brackets give the volume of liquid held when the soil was wetted in air. The other figures are for the soil wetted *in vacuo*.

Again it is seen that the variations between hydrocarbons are small though probably outside experimental error. Wetting the soil under vacuum barely affects the volume of liquid held by Rothamsted soils but it definitely increases the volume held by the Gezira and Gloucester soils. The effect is probably due to the difference in crumb structure of the two sets of soils.

The swelling of the soils in each liquid was also determined in the Keen-Raczkowski box experiment. For ease of calculation the swelling has been defined as the weight of oven-dry soil pushed above the level of the box per 100 gm. of oven-dry soil initially in the box. Table III gives these swellings when the soils were wetted with the hydrocarbons in the presence of dry air at atmospheric pressure.

Table III. *The swelling of the soils in hydrocarbons when wetted in air.*

	Barnfield surface	Barnfield subsoil	Gezira	Gloucester
Paraffin	6.0	5.6	6.6	2.9
Tetralin	7.5	10.0	12.0	3.6
Tetralin (purified)	8.1	10.5	12.4	5.0
Xylene (rectified)	5.9	5.7	7.2	5.0
Carbon tetrachloride (A.R.)	3.9	6.8	1.7	1.6
Benzene (A.R.)	7.6	11.5	12.6	8.6
Hexane (B.P. 67–69°)	3.3	3.6	5.3	1.3

The swellings are seen to be considerable, but they are due entirely to adsorbed and entrapped air, for when the soil is wetted very slowly in a good vacuum the swelling is found to be very small, varying erratically between 0–1 per cent.

THE DISCUSSION OF THE EXPERIMENTAL RESULTS.

As pointed out in the introduction, four assumptions have to be made in determining the imbibitional water held by a soil. The first assumption is that the soil colloids do not imbibe hydrocarbons. The data presented do not prove or disprove this, but they show that if the colloids imbibe hydrocarbons they imbibe all those investigated to approximately the same extent, for the volume of hydrocarbon a soil can hold either in the centrifuge or in the box is nearly independent of the hydrocarbon used. Again, the soils do not swell when wetted with a hydrocarbon *in vacuo*. If a soil swells on wetting *in vacuo* it must imbibe some of the wetting liquid, and, alternatively, if the soil does not imbibe any liquid it cannot swell on wetting. One cannot argue, however, that because a soil does not swell on wetting it does not imbibe any of the liquid, since the imbibed liquid could fill all the micropores and even the macropores without any swelling taking place. Hence all that can be argued is that the results are in accord with the hypothesis that the soils do not imbibe any of the hydrocarbons used.

The second assumption made in the determination of imbibitional water by Fisher's method is that the imbibition of water is effectively complete under a centrifugal field of 1000 g. It is only possible to test the truth of this assumption by using the other three assumptions, namely that the soil colloids do not imbibe hydrocarbons, that the density of a liquid in the soil pores is not altered and that the volumes of water and hydrocarbon held in the soil pores under comparable conditions are equal. Under these conditions the difference between the volume of water and the volume of hydrocarbon held by the soil under comparable conditions will give the *weight* of water imbibed by the soil colloids, for these volumes were obtained by dividing the observed weight of liquid held by its bulk density. Table IV gives the weight of water imbibed by the soils in the centrifuge and in the box experiments determined by this method.

Table IV. *Imbibitional water determined from the centrifuge and from the box measurements.*

(Weight of water imbibed per 100 gm. of soil.)

	Barnfield surface	Barnfield subsoil	Gezira	Gloucester
Centrifuge	10.8	19.9	30.1	16.2
Box	4.7	20.7	31.9	13.4
Centrifuge - Box	6.1	- 0.8	- 1.8	2.8

With the exception of the Barnfield surface soil, which appears to show a much higher imbibition under the centrifugal field, the weight of water imbibed by the soils is within experimental error the same in the two experiments, showing that under a centrifugal field of 1000 g. the imbibition of water is complete.

No direct evidence is given by the data bearing either on the density of liquids in the fine crumb capillaries, or on the volume changes in the micropore space due to the imbibition water. The experiments do show that the macropore space of the soil is unaffected by this imbibition, when the macropore space is defined as the volume of the capillaries containing air under a centrifugal field of 1000 g. This can be measured by the difference between the volume of a liquid held in the box and in the centrifuge, provided no secondary effects enter. There is no evidence that any such effects enter for soils wetted with hydrocarbons or for silts wetted with water, but it is not yet possible to determine directly if any effects enter when the soil swells on wetting. Table V shows that the macropore space is the same within the limits of experimental error for all the wetting liquids used and for all the soils except the Barnfield surface soil.

Table V. *The macropore space of the soils.*

	(c.c. per 100 gm. of oven-dry soil.)				
	Barnfield surface	Barnfield subsoil	Gezira	Gloucester	Mean
Water	17.1	22.1	41.4	32.7	28.3
Paraffin	23.0	20.1	37.5	37.6	29.5
Tetralin (purified)	23.4	21.0	42.1	35.0	30.4
Decalin (purified)	22.8	21.0	40.7	33.1	29.4
Xylene (rectified)	22.1	24.4	40.4	33.9	30.2
Mean of the 4 hydrocarbons	22.8	21.6	40.2	34.9	29.9

THE SWELLING OF SOILS IN WATER.

It is not possible on the experimental results so far discussed to derive any further evidence concerning the validity of the assumptions necessary for calculating the *weight* of water imbibed by a given weight of soil. An estimate of the *volume* of water imbibed by a given weight of soil can, however, be made from the swelling of the soil in the Keen-Raczkowski box. Let a box of volume V c.c. hold W gm. of oven-dry soil, and let σ be the apparent density of the oven-dry soil, where $\sigma = W/V$. On wetting the soil *in vacuo* let W_s gm. of oven-dry soil be expelled above the level of the box and let W_r gm. remain in the box ($W = W_r + W_s$). Now W_r gm. have swollen from W_r/σ c.c. in the oven-dry condition to W/σ c.c. in the

saturated condition, so that the swelling per gram of oven-dry soil is $W_s/\sigma W_r$ c.c. This equals the volume of liquid imbibed by 1 gm. of soil on the assumption that the volume of free water held by the soil in the box equals the volume of air in oven-dry soil, for on this assumption if a soil imbibes v c.c. of water it must swell by v c.c. Thus this method of determining imbibitional water requires, in effect, only the fourth assumption made in Fisher's method.

The mean values of $W_s/\sigma W_r$, the swelling per gram of oven-dry soil, are given in the first row of Table VI. The individual determinations of the swelling are subject to very large variations from one experiment to another, so that $W_s/\sigma W_r$ must be dependent on some unstandardised conditions occurring, of which variations in the closeness of packing of the soil are probably the most important. If the closeness of packing can affect the swelling, the fundamental assumption of this section, namely that the volume of free water held by the wet soil equals the volume of air in the oven-dry soil, can be only partially true.

The volume of the water imbibed by the soil, calculated by this method, is smaller than the weight of water imbibed, as is seen by comparing the top line of Table VI with Table IV.

Table VI. *Volume of imbibitional water held by a soil and its mean density.*

	Barnfield surface	Barnfield subsoil	Gezira	Gloucester
Volume in c.c. per 100 gm. of soil	4.1	17.9	28.2	12.8
Mean density of water = $\frac{\text{weight of imbibed water}}{\text{volume of imbibed water}}$	1.15*	1.13	1.10	1.15

* Using box data only.

If this difference is not due to an incorrect method being used to calculate the weight or the volume of the imbibed water, it must be attributed to the difference in density between free and imbibed water. The density of the imbibed water necessary to make the determinations of the volume and the weight of imbibed water consistent is given in the second row of Table VI and is found by dividing the weight of imbibed water by its volume. The weight used was the mean of the centrifuge and the box values given in Table IV, except in the case of the Barnfield surface soil, for which the box value was used.

It is not possible to decide from direct determinations if the densities of the imbibed water calculated in this way are correct, since no suitable

methods are yet available. Evidence for the correctness of these values can, however, be obtained from the weight of water imbibed and the difference in the specific volumes of the soil in a hydrocarbon and water. Let a specific gravity bottle of volume V contain m gm. of oven-dry soil of specific volume σ and W gm. of water. Let the soil imbibe i gm. of water of mean specific volume δ per gram of soil, so that there are only $(W - mi)$ grams of free water of specific volume ρ in the bottle. Then the apparent specific volume Σ of the soil in water, obtained by neglecting the imbibed water, is clearly

$$\Sigma = \frac{V - W\rho}{m}.$$

If the imbibed water is allowed for, σ , the specific volume of the dry soil is given by

$$\sigma = \frac{V - mi\delta - \rho(W - mi)}{m},$$

so that on eliminating V , W and m from these equations it is found that

$$(\sigma - \Sigma) = i(\rho - \delta) \quad \dots\dots(1).$$

In this equation Σ , the apparent specific volume of the soil in water, i the weight of imbibed water and ρ the specific volume of water, can all be determined directly, but σ , the true specific volume of the dry soil, and δ , the specific volume of the imbibed water, cannot. But the specific volume of a dry soil in paraffin or other non-polar hydrocarbons will be the same as the specific volume of the dry soil in air if the paraffin or non-polar hydrocarbon has the following three properties:

- (1) They are not imbibed by the soil colloids.
- (2) They can penetrate into all the fine soil capillaries and so can wet the soil completely.
- (3) Their density in the fine soil capillaries is the same as their bulk density.

All these properties are already postulated by the methods used to determine the weight of water imbibed by the soil. Thus, if this equality of the specific volume of the dry soil in air and in paraffin is assumed, σ is known so that δ , the mean specific volume of the imbibed water, can be calculated from equation (1).

The specific volumes of the four soils were determined in water and paraffin using the technique developed by one of the authors(5), which allows the soil to be wetted slowly in a vacuum produced by a rotary Hyvac oil pump. The results of these determinations are given in Table VII. For the determination of the mean density of the imbi-

tional water, the value used for the weight of water imbibed was the mean of the centrifuge and box values given in Table IV, except for the Barnfield surface soil. Evidently the values required for the mean density of the imbibed water to bring the determinations of the volume and weight of water imbibed into harmony are reasonably well supported by

Table VII. *The mean density of imbibed water.*

	Barnfield surface	Barnfield subsoil	Gezira	Gloucester
Specific volume in paraffin	0.38891	0.37489	0.37839	0.40010
Specific volume in water	0.38160	0.36626	0.36369	0.38500
Difference = $\sigma - \Sigma$	0.00731	0.00863	0.01470	0.01510
$1/\delta$ = mean density of imbibed water	1.18*	1.04	1.05	1.11
Mean density calculated from swelling (Table VI)	1.15*	1.13	1.10	1.15
Calculated volume of imbibitional water from:				
Centrifuge	10.1	19.0	28.6	14.7
Box	4.0	19.8	30.4	11.9
Observed volume of imbibitional water (Table VI)	4.1	17.9	28.2	12.8

* Imbibitional water determined in box.

the indirect determination of this density from the reduction of specific volume of the soil in water from that in paraffin. At the bottom of Table VII are given the volumes of water imbibed, calculated from the weight of water imbibed when determined either in the centrifuge or in the box, and from the specific gravity determinations. These values can easily be written down by using equation (1) when it is rewritten as

$$i\delta = i - (\sigma - \Sigma),$$

for $i\delta$ is the volume of water imbibed per gram of soil. This agreement between the volume of imbibitional water determined from the swelling of the soil in the Keen-Raczowski box and the volume calculated from the weights of water hydrocarbon held by the soil in the moisture equivalent centrifuge and the difference in specific volume of the soil in water and the hydrocarbon does lend strong support to the interpretations of the measurements which have been given here. The exceptional case of the Barnfield surface soil, however, does suggest that some other factor besides those discussed here may be important under certain conditions.

CONCLUSIONS.

Two related methods of determining the weight of water imbibed by a soil and one independent method of determining the volume of water imbibed have been discussed, and the three methods have been shown to give concordant results. The first two methods are based on the four assumptions:

- (1) That soils do not imbibe hydrocarbons.
- (2) The density of the liquid in the fine soil pores is the same as the bulk density of the liquid.
- (3) The volume of hydrocarbon held by a dry soil equals the volume of air held by the dry soil.
- (4) The volume of free water held by a wet soil equals the volume of air held by the dry soil.

For the determination of the volume of the imbibed water it is only necessary to make the last assumption.

The determinations on the weight, volume, and density of the imbibed water by these different methods are reasonably concordant for three of the soils. One of the methods does, however, give discrepant results for the Barnfield surface soil, a soil which contains large quantities of readily decomposable organic matter, for it has had 14 tons of farmyard manure applied to it annually for over 70 years. It is, of course, not possible to argue that because the determinations of the weight and volume of the imbibed water are concordant therefore all the assumptions are valid, though the validity of the first three assumptions, which are only required for one of the determinations, is rendered more probable.

The fourth assumption, however, is probably only partially true, for the amount of swelling of these soils in water appears to be sensitive to some uncontrolled factor, which is probably the closeness of packing. It is unlikely that any air can be entrapped during wetting, causing these differences in swelling, since the wetting technique appears to allow the escape of all entrapped and adsorbed air.

The tentative conclusion arrived at from these results is that the weight or volume of the water imbibed by a soil can be measured. The main limitation in this discussion has been, however, that the accuracy of the different methods is not sufficiently great to allow a consideration of the small differences usually found between one method and another.

SUMMARY.

Two methods of determining the weight and one method of determining the volume of water imbibed by a soil have been discussed. The results of these methods have been shown to be concordant by an independent method.

The tentative conclusion reached is that the weight of water a soil imbibes can be readily determined by Fisher's method or from the Keen-Raczkowski box, but that the volume of imbibed water cannot yet be directly determined accurately.

REFERENCES.

- (1) RUSSELL, E. W. *J. Agric. Sci.* (1933), **23**, 261.
- (2) FISHER, E. A. *J. Agric. Sci.* (1924), **14**, 204.
- (3) COUTTS, J. R. H. *J. Agric. Sci.* (1932), **22**, 203.
- (4) — *J. Agric. Sci.* (1930), **20**, 407.
- (5) RUSSELL, E. W. (In the press.)

(Received February 7th, 1934.)

THE pF OF THE WATER IN SOIL

R. K. SCHOFIELD

(Rothamsted Experimental Station, Harpenden, England)

As this paper is mainly devoted to a discussion of soil moisture relationships from a new standpoint, and in terms of a new scale which, for reasons given, I have called the pF scale, there is only space for the briefest summary of the very large number of earlier investigations which have had as their object the furthering of our knowledge of the laws of soil water movement. Very broadly it may be said that these investigations have fallen into three main groups.

In one, the known laws of viscosity and surface tension have provided the starting point.* The principles of viscous flow have, for instance, been applied with considerable success to the movements of water in porous soils below the water-table. Moreover, using the well-known formula for the pressure difference across a curved meniscus, deductions have been made about the distribution of water above a water-table which agree with the observed behaviour of sand. It is not to be expected that these deductions would apply quantitatively to clay soils, because it has long been recognised that all very finely divided materials show a characteristic behaviour towards water which, together with allied properties, has caused them to be described as colloidal. Actually serious departures are observed with all soils containing an appreciable number of particles below about 0.02 mm.—that is to say, with practically all agricultural soils.

Our lack of exact knowledge of colloidal behaviour makes it difficult to proceed deductively in the case of ordinary soils. Consequently, in a second group of investigations, the inductive method has been followed. In particular a number of investigators in the western states of the U.S.A.† have shown that a limited amount of water applied at the surface of a deep uniform layer of relatively dry soil of medium texture, wets the soil nearly uniformly to a certain depth, and that this wet layer does not give up more than a small fraction of its moisture by downward movement into the relatively dry layer below. Though this is by no means the only important fact which has come to light through direct observations and experiments on soil, it has such obvious significance that it provides a fair test to apply to any approach of a theoretical kind that may be proposed. The surface tension theory explains quite satisfactorily the behaviour of sands which, in striking contrast to ordinary soils, allow water to pass through them to the water-table leaving the surface layers little or no wetter than they were to start with, but appears incapable of accounting for the distribution described above.

The few who form the third group have endeavoured to work out energy relationships which should hold whatever the mechanism at work, and which should, therefore, be applicable to ordinary soils as well as pure sands. The advantages of approaching the problem in this way are more evident now than when Buckingham (5) initiated the attack, and it is rather surprising that Gardner (6) and his associates in Utah alone have hitherto followed the lead. It is true that Gardner

*See Keen, "The Physical Properties of the Soil," Chapter III. (Longman).
Alway (1), Israelsen (2), Shaw (3) and Veihmeyer (4), to mention but four.

failed to account for the moisture distribution described above, but reasons are given in this paper for believing that this was due, not to unsoundness in the underlying principles, but to lack of appreciation of the fact that the soil moisture conditions which are of most practical interest and importance are not conditions of true equilibrium.

The position is most easily explained without the use of technical language by reference to a simple experiment. The first part resembles one described by Bouyoucos (7) in which water is removed from a small mass of wet soil by placing it on a Buchner funnel. The chief difference is that a suspension of a fine silt is poured on the filter paper before the soil is applied. The silt forms a thin layer (1 mm. or less in thickness) which allows water to pass readily, but through which air will not pass, even under the full pressure of a suction pump. By this means it is possible to maintain any desired pressure difference up to one atmosphere across the filter, and measure it on a manometer. With this arrangement it is a simple matter to determine how the moisture percentage retained by a soil sample previously very wet, depends on the pressure difference applied.

In the second part of the experiment a dry layer of soil is spread over the silt layer after the pressure in the filter flask has been adjusted to the desired value. The soil layer is held in place by fitting a rubber bung (not air-tight) into the top of the funnel. The funnel and flask are then inverted, enough water having previously been allowed to accumulate in the flask for some to pour down the stem of the funnel, and stand above the filter paper. In this way it is possible to determine how much water a layer of dry soil absorbs against a known pressure difference. This experiment is similar in principle to many described in the literature in which porous candles full of water and attached to manometers have been buried in soil; but, in addition to the employment of a silt layer, it is distinguished by the use of thin layers of soil (a few millimetres only). It is obvious that the thinner the layer, the sooner will a uniform moisture content be attained which can then be determined as corresponding to the pressure difference used.

The purpose of the experiment was to demonstrate by very simple means that the moisture content to which an ordinary agricultural soil can be dried by a given suction is considerably greater than that to which it can be wetted against the same suction, in any reasonable length of time. The same conclusion can be inferred from the observations of the American workers already referred to, for the fact that a soil layer at, say, 20 per cent. moisture can lie close to another layer at, say, 12 per cent. moisture, and water only pass extremely slowly between them shows that a dry layer when taking up moisture, even very slowly, can only exert a suction sufficient to abstract water from a much wetter layer.

Thus we see that, although the assumption of Gardner (also made by Buckingham) that a soil exerts a definite equilibrium suction depending only on its moisture content may be true in relation to geological time, in practice this suction depends very much on whether the soil is wetting or drying. To a lesser degree it is undoubtedly affected by a number of other factors, many of which can be included under the heading of "history." Nevertheless, it seems well worth enquiring how far the practical problems of soil moisture movement

can be elucidated by making the simple assumption (admittedly only a first approximation) that for each moisture content there are two characteristic suctions, one for drying and the other for wetting.

In prosecuting this enquiry it has proved convenient to use a new scale, which I have called the pF scale, to express what has, in the previous pages, been called "suction." Buckingham called it "capillary potential" and defined it as the height in centimetres of the equivalent water column. I have deliberately not used this term because the word "capillary" brings to so many minds thoughts about surface tension. The great virtue of energy relationships is that they hold irrespective of particular mechanisms.

The pF is the logarithm of Buckingham's potential. By analogy with Sørensen's acidity scale the symbol "p" indicates its logarithmic character, while the symbol "F" is intended to remind us that by defining pF as the logarithm of the height in centimetres of the water column needed to give the suction in question, we are really using the logarithm of a free energy difference measured on a gravity scale. By basing our scale on free energy rather than pressure, we are not troubled in our calculations by the influence of pressure on the density of water.* We can also transfer the scale to any liquid, defining its pF as the logarithm of the height of a column of that liquid.

The suction given by a Buchner funnel obviously cannot exceed one atmosphere or 1000 cm. of water; so that pF 3 marks the limit for such experiments as have already been described. Higher values could presumably be reached by using air pressure in conjunction with a suitable filter, but in the absence of any measurements of this kind they must be investigated by other means. Measurements of freezing point and vapour pressure are of use here. The highest temperature at which ice crystals will form and remain in a moist soil is lower than 0° C., because the free energy of the water in soil is less than that of water in bulk under the same atmospheric pressure. The free energy difference (and hence its logarithm the pF) is directly related to the freezing point depression. For the same reason the pressure of water vapour over a moist soil is less than that over water in bulk at the same temperature. The formulæ for calculating the pF from freezing point depressions and relative humidities are derived below.

Freezing Point. *
$$\begin{aligned} H &= (T_g/L_j) \times t. \\ pF - \log_{10} H &= \log_{10} (T_g/L_j) + \log_{10} t. \\ &= 4.1 + \log_{10} t \text{ for water at freezing point.} \end{aligned}$$

Vapour Pressure (Relative Humidity).

$$\begin{aligned} H &= -(RT/Mg) \log_e(h/100) = +2.303(RT/Mg) \log_{10}(100/h). \\ pF &= \log_{10} H = \log_{10} 2.303(RT/Mg) + \log_{10}(2 - \log_{10} h). \\ &= 6.5 + \log_{10}(2 - \log_{10} h) \text{ for water at } 20^\circ\text{C.} \end{aligned}$$

H = height of liquid column to give equivalent suction, cm.

T = absolute temperature, °C.

g = gravitational acceleration, 981 ergs. gm.⁻¹ cm.⁻¹.

Lj = latent heat of fusion, 3.336. 10⁹ ergs. gm.⁻¹ for water.

t = freezing point depression, °C.

R = universal gas constant 8.315. 10⁷ ergs. moles.⁻¹ °C.⁻¹.

T = absolute temperature, °C.

M = molecular weight in vapour phase, 18.02 gm. moles.⁻¹ for water.

h = relative humidity per cent.

*In the equation $\Delta F = \int v dp$, v is the sp. volume, p the pressure and ΔF the free energy change corresponding to the pressure difference dp.

Through the use of a logarithmic scale it is possible to show the variation of suction over the whole moisture range on one diagram, thereby making light of the fact that the equivalent water column at 50 per cent. relative humidity is almost 10 kilometres (pF 6) or higher than Mount Everest, while that for oven dryness is nearly ten times greater (pF 7).^{*} Accurate vapour pressure data are difficult to obtain below about pF 4.15 (99 per cent. relative humidity), so it is fortunate that freezing point measurements are possible between about pF 4.4

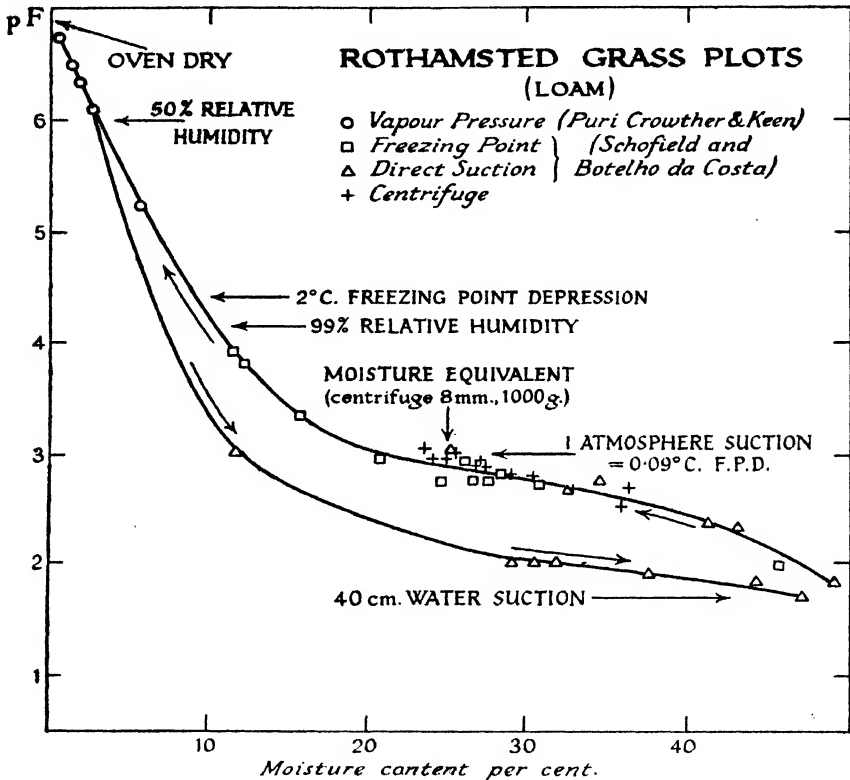


FIG. 1.

(2° C., F.P.D.) and pF 3 (0.09° C., F.P.D.), below which direct suction measurements can be made.

Data for a sample of soil from the Rothamsted Grass Plots are plotted in Fig. 1 by way of illustration. The suction and freezing point measurements were made with the assistance of Mr. J. V. Botelho da Costa. The freezing point measurements only give points on the upper branch since freezing is essentially a drying process. As vapour pressure measurements for this soil had not been completed at the

^{*} Assuming 1 per cent. moisture in the air of the oven, and taking 0.01 units as the decrease in pF for 20°C. rise in temperature (average of few existing data), the following are provisional figures for the pF of soils at 20°C. after oven drying:—

Temperature of oven.	100°C.	105°C.	110°C.
pF at 20°C.	6.91	6.93	6.95

time of writing, the corresponding points on *Fig. 1* are for a very similar soil investigated by Puri, Crowther and Keen. The centrifuge points will be discussed later.

The soil samples that were dried by suction had all been soaked for 48 hours and the controlled suction was applied for times varying from 1 to 24 hours. Lengthening the time of suction may have reduced the moisture contents a little, but there were irregularities of the same order between samples apparently treated alike. Pending further investigation it seems reasonable to ascribe to the difficulty of accurately reproducing the pretreatment both the scatter of the suction points and their tendency to lie a little above those obtained by freezing. This scatter draws attention to the fact that it is only to a first approximation that pF and moisture content for drying conditions can be considered to be connected by a single curve. The scatter of the points is, however, small in comparison with their separation from those obtained by wetting.

When wetting against suctions less than pF 2, 24 hours appeared to be ample for the attainment of a reasonably steady moisture content. At pF 3 it may well be that the time was insufficient notwithstanding the thinness of the soil layer, as wetting was observed to be very slow. The moisture content plotted for pF 3 on the wetting curve may, therefore, be somewhat low. There is no doubt, however, that over the central portion of the moisture range (that of most importance in the field) the suction this soil can exert in taking up moisture is only about one quarter that required to withdraw water from it at the same moisture content.

A number of less accurate means for determining pF values are also available. Shull (8) devised an ingenious way of determining suction pressures up to hundreds of atmospheres by using xanthium seeds. These have semi-permeable coats which admit water but not salts, so that the moisture content of these seeds adjusts itself to the free energy of the water of any solution into which they are placed. After "calibration" in solutions of known properties these seeds can be used to measure the free energy of the water in soils. Though interesting, the method is not of great practical importance, as it rests ultimately on vapour pressure and freezing point determinations made on the solutions used in the calibration, and is much less accurate than either of these methods when applied direct to the soil.

Buckingham considered that the distribution of moisture in a vertical column of soil would give the relationship between moisture content and capillary potential. Even apart from the importance of knowing the direction in which the moisture content is changing, the position in this case is complicated by the increase in pressure in passing from the top to the bottom of the column. The experiments of Terzaghi (9) have shown that, when compressed in cylinders with porous ends, clays may be made to part with considerable amounts of water, some of which will return when the pressure is released. The effect is very small in sands. Nevertheless, in general for ordinary soils the height from the water-table and the effect of pressure have opposite influences on the moisture content. In the case of very heavy soils the effect of pressure may outweigh that of height, and cause the moisture content to decrease with depth. Only the top layer

is uninfluenced by pressure. If this is protected from evaporation it should presumably have the same moisture content as an isolated layer of the same soil under an equivalent suction, and in the dynamic condition (wetting or drying).

The use of a tall column is, however, most inconvenient, owing to the very long time needed to reach a final distribution. This difficulty is overcome by using short columns in a high centrifugal field. Veihmeyer, Israelsen and Conrad (10) have shown that a layer of a medium loam 1.6 cm. thick, after being centrifuged in a field equivalent to 1,000 times gravity, varied very little in moisture content from the inner to the outer surface. Consequently the moisture content found by oven-drying the whole sample would not have been very different from that of the innermost layer to which alone the value $pF\ 3.2$ (log 1,600) strictly applies. The close correspondence seen in Fig. 1 between the points for suction and freezing point and those for centrifuge measurements shows that the soil of the Rothamsted Grass Plots behaves similarly. The moisture contents in the case of the centrifuge determinations are for the whole of each 30 gm. sample (average thickness 8 mm.) while the pF values are calculated for the innermost layer from the speed of the centrifuge. This equivalence cannot be expected to hold in the case either of very heavy or very light soils; but if no better data are available the moisture equivalent determined with a 30 gm. sample in a standard Briggs-McLane centrifuge (1,000g) may be taken as about $pF\ 2.9$ on the drying curve for medium textured soils. With a centrifuge giving a field of 70,000g, Lebedeff (11) reduced the moisture content of soil samples to about the same extent as freezing at -1.5°C . ($pF\ 4.28$). Assuming his soil layer averaged 3 mm. in thickness, the inner surfaces were subjected to a suction amounting to $pF\ 4.33$. He found almost the same moisture content at 27,000g ($pF\ 3.9$) but the rather serious drying caused by air currents makes exact comparison difficult. Nevertheless, his "maximum molecular moisture-holding capacity" may be taken as a point on the drying curve round about $pF\ 4$.

The amount of water which another absorbent material can remove from a soil, or a soil can remove from another absorbent material, will depend on a number of factors including, respectively, the drying and wetting pF of the soil at the final moisture content. By "calibrating" an absorber against soils for which the pF moisture characteristics have been found by absolute methods, it is possible to obtain at least a portion of the pF -moisture curve of other soil samples, and under certain conditions to determine pF values for field soils *in situ*. Some years ago, E. M. Crowther in unpublished studies at Rothamsted (mentioned here by kind permission), placed wet soil samples on plates of unglazed earthenware (crystal driers), with the idea that the soils would be dried to an extent that would characterise their moisture-holding power. Some of these dishes have now been calibrated and it appears that their moisture content falls off very rapidly between $pF\ 3$ and $pF\ 4.5$, with little further loss to the oven-dry state. Experiments are now in progress which, it is hoped, will show that these plates may be used for rapid approximate determinations of pF within this range. This rapid change in moisture content is probably also a property of the unglazed earthenware "soil

points" introduced by Livingstone (12). If calibrated, their uptake of moisture from soil in the field could be interpreted in terms of pF. (See below.)

In view of the many ways in which pF values can be determined, it is not surprising that a large amount of data exists from which portions of drying and wetting curves can be plotted. Amongst these the freezing point measurements of Parker (13) are of particular interest, as benzene and nitrobenzene were used in addition to water. The soil was dried at 105° C. for twelve hours before use. The

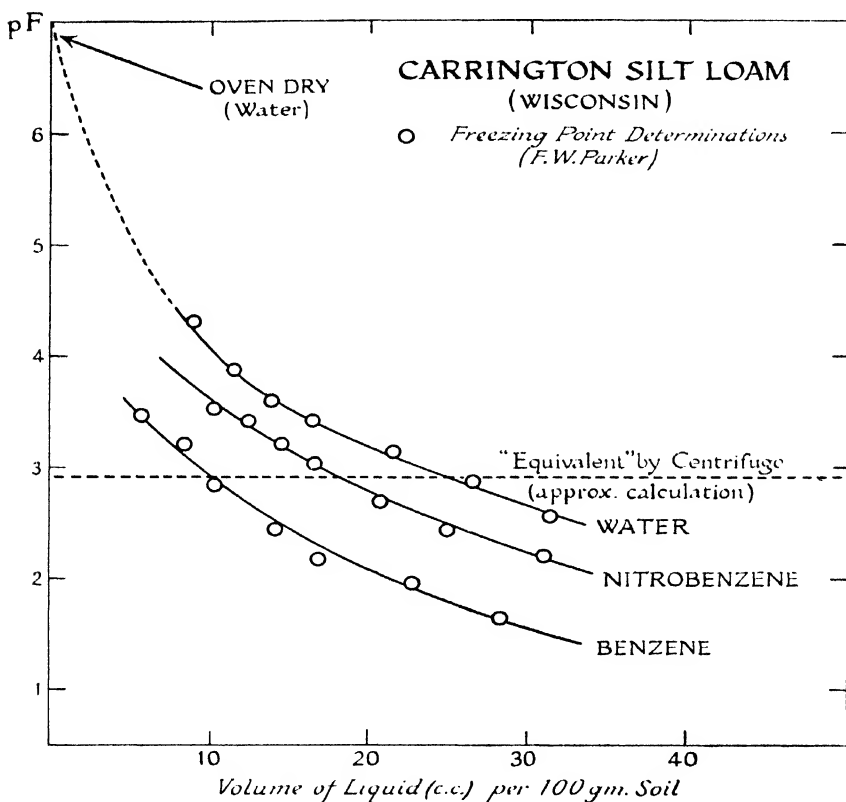


FIG. 2.

curves (Fig. 2) illustrate the power of the pF scale to set out the facts in an orderly fashion, for no regularity was noted in the original paper. They also suggest that the difference in the behaviour of this soil towards the three liquids is one of degree rather than kind.

The only soil, besides the one from Rothamsted already discussed, for which it is possible to trace the greater part of both curves, is that of the Greenville Farm of the Utah Experiment Station. Thomas (14) gives vapour pressure and centrifuge data, and Hilgard maximum moisture for one sample, while Richards (15) gives data for wetting

against suction through a porous plate. The combined data, expressed on the pF scale, are plotted in Fig. 3.

Thomas found that beans wilted permanently in this soil when they had reduced its moisture content to 9.3 per cent., which the drying curve shows to correspond to pF 4.25. Determinations by vapour pressure, freezing point and seed absorption agree* in giving a figure close to pF 4.2 for all soils so far examined. The recent work of Veihmeyer and Hendrickson confirms the original conclusion of Briggs and Shantz (16), that plants in great variety act similarly

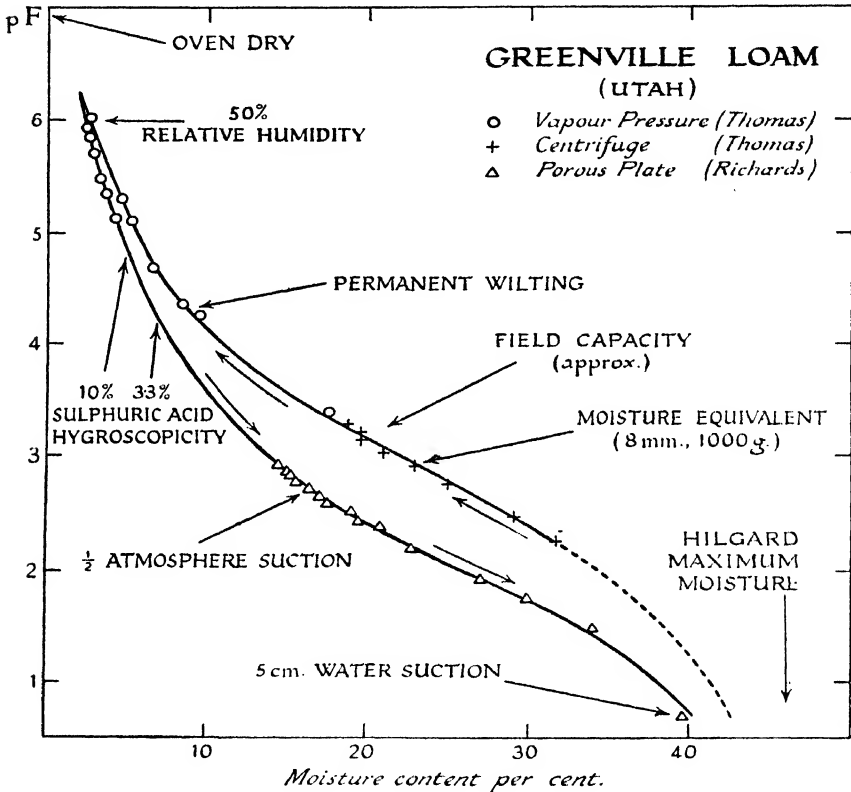


FIG. 3.

in this respect, and that the result is not significantly influenced by environmental conditions, except possibly when these are very extreme. Although more work is needed in this field, existing evidence points to the conclusion that a determination of the moisture content on the drying curve at pF 4.2 would give nearly enough for practical purposes that for permanent wilting.

Much confusion has arisen in the past from the use of the indirect methods proposed by Briggs and Shantz for calculating the "wilting coefficient." For whereas they considered that a sufficiently accurate figure might be obtained by dividing the moisture equivalent by 1.84,

*See Schofield and Botelho da Costa, ref. (18).

Veihmeyer and Hendrickson (17) have found the true ratio to vary from 1.4 to 3.8. This variation arises from the fact that the drying curves of soils differ considerably in shape, which is well illustrated by the freezing point measurements of Schofield and Botelho da Costa (18), and may also be seen by comparing *Figs. 1 and 3*. Nor is the moisture content at permanent wilting obtainable with sufficient accuracy by multiplying the "hygroscopic coefficient" by a constant factor. Even if we take the moisture content reached by a soil, initially air dry, placed over 3.3 per cent. sulphuric acid, by which process it is brought to pF 4.2, this is on the wetting curve. The vapour pressure measurements of Thomas show that the ratios of the moisture contents at pF 4.2 on the two curves differ considerably from soil to soil. The same may be inferred from the dilatometer experiments of Bouyoucos (19). The amount of water which failed to freeze on first cooling to -1.5° C. (pF 4.28) was from 1.0 to 1.7 of that which was unfrozen on warming from -78° C. to -1.5° C. Veihmeyer and Hendrickson found the same range of variation for the ratio of the moisture content and permanent wilting to the hygroscopicity over 3.3 per cent. sulphuric acid. In neither case was there any obvious connection between this ratio and other characteristics of the soil. Attempts to connect the moisture content at permanent wilting with mechanical analysis have been no more successful, so a direct determination of the moisture content at pF 4.2 on the drying curve seems to be the only alternative to a wilting test.

If, after a soil like that of the Greenville Farm has been allowed to dry through root action to a considerable depth, a limited amount of water is applied, two stages can be distinguished. During the application water runs fairly quickly through the structural pores and cavities of the soil and is absorbed by the soil material before it has penetrated far. The condition during this stage is represented by a point on the wetting curve having a very low pF. Soon after the application ceases there is no longer any water free to run through the structural pores under the action of gravity. It has all been taken up by the soil material in a wet layer extending from the surface to a depth depending on the amount of water applied. Further downward movement into the relatively dry soil below must involve a drying of the upper wet layer. During this second stage the pF is much higher, as its relation to the moisture content of the upper layer is now given by the drying curve. Movement of water into the drier lower layer where wetting conditions still obtain must now be made against a suction from the upper layer which rises more and more as water is withdrawn from it. As already noted in connection with the simple suction experiment described earlier, it was found that a comparatively steady moisture content was reached rather slowly by wetting at higher pF values. In harmony with this it is the observation of the American investigators that after a time downward movement becomes very slow. Thus the effective limit to which the moisture content of the upper layer can be reduced by the downward movement of water appears to be set by the pF value above which the wetting of the dry soil is too slow to be of practical importance. As it has generally been found that the limiting moisture content or "field capacity" is not very different from the moisture equivalent, wetting must become very slow about pF 3, so that the upper layer is left at the

corresponding point on the drying curve. It is probable that the pF at a given time after the application ceases is rather higher for heavy than for light soils.

Many cases will not be as straightforward as the one just considered. Impeded drainage may arrest the process in the first stage. The presence of a very sandy layer, although giving free drainage (pF 1 or below), is a barrier to the movement of water above about pF 2 (the exact limit depending on the grade of sand). Less drastic changes of texture in the profile will lead to variations in the moisture content in the wet layer, even though it has practically the same pF throughout. There will, of course, be no second stage where enough water is added to drain to the water-table, as in winter conditions in England. Moreover, even in English summer conditions, desiccation rarely goes far enough and deep enough for the second stage to be fully developed.

In all cases, however, we can apply the principle that water will not move except where there is a pF gradient (due allowance being made for gravity). Even when a pF gradient exists, movement may be slow due to low permeability, but it is important always to bear in mind that a moisture gradient does not necessarily involve a pF gradient. A direct determination of the pF at any point in a field soil will not always be easy. In the root zone of actively transpiring plants we have drying conditions, and a dry soil point will take up an amount of moisture corresponding to the pF ruling in the soil before it was introduced. A dry soil point would not, however, give the pF of a soil layer that was being wetted, or in which the latest moisture change had been in the direction of an increase. A porous pot with manometer attached may impose wetting conditions when buried in a soil where roots are not active. A subsequent rise in the observed suction with the approach of active roots is an indication of the onset of drying conditions rather than a sign that much water has been taken from the soil.

It was said a little earlier that when a dry soil is wetted, water is absorbed by the soil material. No particular mechanism was implied in using this expression since pF values can be determined and discussed without any reference to the mechanism at work. At the same time, it is clear that the moisture content of a dry soil is able to increase not only because water can fill pores of dimensions fixed by the size and packing of the soil particles, but also because it can make room for itself by separating the particles a little from one another, and causing the mass to swell. The second process has evidently much in common with the building up of layers of liquid many molecules deep on solid surfaces from vapours below the saturation pressure. There is an important difference, however, in that the measurements of McHaffie and Lehnier (20) have shown this deposition on the surfaces of glass, silica and platinum vessels to be reversible: *i.e.*, the thickness of the layer for a given relative vapour pressure is the same for wetting and drying. The water which causes dry soil to swell must move through narrow passages, and moreover, cause relative movement of the particles not only perpendicular to their surfaces, but, on account of their different sizes, tangentially as well. It is known that plastic movements generally are not strictly

reversible, but are subject to hysteresis, so we should not be surprised to find that the same is true of the micro-plastic movements involved in the swelling and shrinking of soil. It seems likely that the difference in the behaviour of soil on wetting and drying is due more to the micro-plastic resistance to swelling and shrinking, than to surface-tension effects such as those discussed by Haines (21). The influence of time as well as extent of wetting or drying on the subsequent behaviour of soil is more easily understood from this point of view, as is also the difficulty of wetting a dry soil with water of high pF.

In connection with plasticity it is natural to enquire whether the characteristic plastic conditions recognised by Atterberg and others occur at definite pF values. In general the answer appears to be in the negative, and hence it may be that a determination of the pF of these characteristic conditions will give additional information. For instance, one of the conditions determining the utilisation of different soils is the rapidity with which they become dry enough after rain to be cultivated. A determination of the pF on drying to the lower plastic limit seems to be a hopeful line of attack here. A measurement of this kind may well prove very helpful in distinguishing clay soils of different kinds, because it involves physical properties of direct influence in the field.

In this brief survey it has only been possible to outline a few of the ways in which the pF scale and the ideas behind it can usefully be applied. Consideration has principally been given to the part of the pF scale that has the most direct bearing on practical soil moisture problems. There are, however, indications from existing data that the course of curves should be considered over as wide a range as possible when considering their value for soil classification. At the dry end the curves appear to reflect the buffer capacity, and are closely connected with heat of wetting and apparent density. In the region of low pF values, which has been little explored up to the present, structure is doubtless a factor of great importance.

One advantage of the point of view here developed is that it enables us to discuss soil moisture relationships in detail, without the use of words of doubtful validity which have been introduced from time to time to describe the different forms in which water has been imagined to exist in the soil. The symbol pF, like its mother pH, has also the merit that it gives no trouble to translators, a point that will surely appeal to members of our international society.

SUMMARY

A treatment of soil moisture relationships based on energy considerations has the advantage that the results obtained are true regardless of the mechanisms at work.

Buckingham's assumption that there is an equilibrium suction for each moisture content does not provide a satisfactory practical basis.

The suction needed to withdraw water from a moist soil is, in general, greater than that against which water will enter the soil at the same moisture content.

In order to deal conveniently with the whole range of suction, use is made of the logarithm of the height in centimetres of the equivalent

water (or other liquid) column. The symbol pF is used for this quantity.

The determination of pF by direct suction, freezing point, vapour pressure, vertical columns, centrifuge and absorbent materials is considered.

It is shown that by carefully distinguishing wetting from drying conditions the results of investigations on plant wilting and field moisture capacity receive a rational interpretation.

It is suggested that in ordinary soils the difference between the behaviour on wetting and drying is due more to micro-plastic resistance to swelling and shrinking, than to surface-tension effects.

Further lines of enquiry are indicated.

REFERENCES

- ¹ ALWAY, F. V., and CLARK, V. L., *25th Ann. Report Nebraska Expt. Sta.*, 1911, p. 246; ALWAY, F. V., and MCDOLE, G. R., *J. Agric. Res.*, 1917, **10**, p. 391.
- ² ISRAELSEN, O. W., *J. Agric. Res.*, 1917, **13**, p. 1.
- ³ SHAW, C. F., *Soil Sci.*, 1927, **23**, p. 303.
- ⁴ VEIHMEYER, F. J., *Hilgardia*, 1927, **2**, p. 125.
- ⁵ BUCKINGHAM, E., *U.S. Dept. Agric. Bur. of Soils*, 1907, *Bull.* 38.
- ⁶ GARDNER, W., *Soil Sci.*, 1919, **7**, pp. 313 and 319; 1920, **10**, pp. 103 and 357; GARDNER, W., and WIDSTOE, J. A., *ibid*, 1921, **11**, p. 215.
- ⁷ BOUYOUCOS, G. J., *Soil Sci.*, 1929, **27**, p. 233.
- ⁸ SHULL, C. A., *Bot. Gaz.*, 1916, **62**, p. 1.
- ⁹ TERZAGHI, C., *1st Int. Cong. Soil Sci.*, Washington, 1927, *Com. V*, p. 127.
- ¹⁰ VEIHMEYER, F. J., ISRAELSEN, O. W., and CONRAD, J. P., *California Agric. Expt. Sta. Tech. Paper* **16**, p. 1.
- ¹¹ LEBEDEFF, A. F., *2nd Int. Cong. Soil Sci.*, Moscow, 1930, *Com. VI*, p. 82.
- ¹² LIVINGSTONE, B. E., and KOKETZU, R., *Soil Sci.*, 1920, **9**, p. 469.
- ¹³ PARKER, F. W., *J. Amer. Chem. Soc.*, 1921, **43**, p. 1011.
- ¹⁴ THOMAS, M. D., *Soil Sci.*, 1921, **11**, p. 409; 1924, **17**, p. 1; 1928, **25**, p. 409.
- ¹⁵ RICHARDS, L. A., *J. Agric. Res.*, 1928, **37**, p. 719.
- ¹⁶ BRIGGS, L. J., and SHANTZ, H. L., *U.S. Dept. Agric. Bur. Plant Ind.*, 1912, *Bull.* 230.
- ¹⁷ VEIHMEYER, F. J., and HENDRICKSON, H., *Plant Phys.*, 1928, **3**, p. 355, and *Amer. Soil Surv. Ass., Bull.* XV, 1934.
- ¹⁸ SCHOFIELD, R. K., and BOTELHO DA COSTA, J. V., *3rd Int. Cong. Soil Sci.*, Oxford, 1935, *Vol. I*, p. 6.
- ¹⁹ BOUYOUCOS, G. J., *J. Agric. Res.*, 1917, **8**, 195, and *Michigan Expt. Sta.*, 1918, *Tech. Bull.* 36.
- ²⁰ McHAFFIE, I. R., and LEHNER, S., *J. Chem. Soc.*, 1925, **127**, p. 1559, and LEHNER, S., *ibid*, 1926, p. 1785, and 1927, p. 272.
- ²¹ HAINES, W. B., *J. Agric. Sci.*, 1930, **20**, 97.

[Reprinted from the *Journal of Agricultural Science*, Vol. XXIV. Part I.]
[All rights reserved.]

* p. 39. For $H = (Tg/Lj) \times t$, read $H = (Lj/Tg) \times t$.
For $pF = \log_{10} H = \log_{10} (Tg/Lj) + \log_{10} t$,
read $pF = \log_{10} H = \log_{10} (Lj/Tg) + \log_{10} t$.

THE USE OF THE GLASS ELECTRODE IN SOIL REACTION AND OXIDATION-REDUCTION POTENTIAL MEASUREMENTS.

By S. G. HEINTZE.

*(Chemistry Department, Rothamsted Experimental Station,
Harpenden, Herts.)*

(With Three Text-figures.)

ALTHOUGH the quinhydrone electrode is generally recognised as the most convenient method for reaction measurements of soil suspensions, it is liable to give erroneous results in many common kinds of soil(1, 2, 3). It is therefore of interest to ascertain the value of alternative electrodes which can serve for independent check measurements, even though they should prove to have no advantage over the quinhydrone electrode where this is known to be reliable. For this reason the following results of experience with the glass electrode are placed on record.

Full discussions of the glass electrode are available in several textbooks, notably that of Clark(4), as well as in a series of papers of MacInnes and co-workers, and it is therefore unnecessary to present the underlying theories or practical details, except in so far as they relate to soils.

The theory of the glass electrode was put forward as early as 1909 by Haber and Klemeniewicz(5), but it was not until 1925 when Kerridge(6) improved the design that the electrode came into more general use, especially for biochemical purposes. More recently the introduction of special valve amplifiers, capable of operating in systems with such high resistances as that provided by the glass membrane, has made it possible to dispense with the troublesome quadrant electrometer and the careful shielding imperative in earlier designs. In the above-mentioned series of papers by MacInnes and Doles(7, 8) on the use of the glass electrode for pH measurements of a high degree of accuracy, it was shown that the glass electrode may be regarded purely as a hydrogen electrode, provided the salt concentration is not too high. Its specific advantages may be summarised as follows. It is independent of oxidants and reductants and has a low salt error. Further, it causes no alteration in the system under measurement. It is thus free from the poisoning effects,

the loss from carbon dioxide, and the reduction of oxides of manganese and other materials which may occur when the hydrogen or the quinhydrone is used for soils. The reproducibility of measurements is much greater than with metallic electrodes as *e.g.* the antimony electrode.

pH MEASUREMENTS ON SOIL SUSPENSIONS.

The electrical measuring apparatus was one designed by Harrison⁽⁹⁾ and supplied by Messrs Kay and Co., 4 Holborn Place, London, W.C. 1, in a convenient unit which was used with a simple Cambridge potentiometer. The chief feature of this device is a specially designed amplifying valve—an Electrometer Triode from Messrs Philips Lamps, Ltd.—which has so low a grid current that there is no change in anode current due to introduction of resistance into the grid circuit. For measurements on soil suspensions and soil crumbs glass electrodes made by Messrs Dixon of 27 Devonshire Street, W.C. 1, have proved very suitable. The thin glass membrane is in the form of a re-entrant bulb in a stout bulb at the end of a short tube (Fig. 1). This arrangement provides great mechanical strength and easy cleaning. As recommended by Harrison, the glass was made by fusing silica 60 per cent., sodium carbonate 30 per cent., and calcium carbonate 10 per cent. The electrode was supported on the tube of a saturated calomel electrode of the Kerridge type carefully insulated through “Amberoid.” All parts of the apparatus were mounted on an earthed copper plate and batteries and vessels containing the soil suspension were placed on large paraffin blocks. No further shielding or insulation proved to be necessary. The glass electrode contained Veibel solution (0.01 N HCl, 0.09 N KCl). The suspension to be measured was placed in a beaker and stirred by means of the glass electrode so that soil particles settled down inside the thin bulb. To complete the circuit a second Kerridge saturated calomel electrode was placed in the suspension at some distance from the bulb. Diffusion of potassium chloride was prevented by fitting the electrodes with ground-glass caps. The

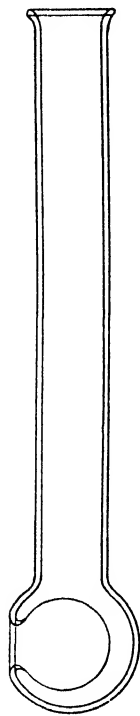


Fig. 1. Length 9.5 cm., diameter of outer bulb 0.9 cm.; diameter of inner bulb 0.6 cm.

30 *The Use of the Glass Electrode in Soil Reaction*

"asymmetry" of the glass membrane and calomel electrodes was small (not more than 2 mv.), but in all cases it was eliminated by referring all measurements to a standard solution. Equilibrium was reached within the first 5 min. in soil suspensions.

The difference in hydrogen and glass electrode potentials of buffer solutions varying from pH 2.5 to 9.5 was constant within the limits of measurements (1 mv.).

In a series of soil samples selected so as to give the widest possible range of type of soil, country of origin, and reaction (pH 3.8-9.1) measurements were so satisfactory that it is not necessary to tabulate typical values. In a series of nineteen soils for which the quinhydrone electrode gave reliable values as shown by independent tests, the mean difference in pH by glass and quinhydrone electrodes was +0.01 and the standard error of a single difference was ± 0.05 . For twelve soils to which the quinhydrone method was not applicable, the mean difference by glass and hydrogen electrodes was 0.00 with a standard error of a single difference of ± 0.04 .

pH MEASUREMENTS ON MOIST SOILS.

The fact that in the glass-electrode method no foreign substance has to be incorporated uniformly throughout the soil suggested the possibility of glass-electrode measurements at lower soil moisture contents than are possible when quinhydrone or hydrogen must be brought into equilibrium with the soil. Crumbs of moist soil, taken directly from the field or prepared by remoistening air-dried soils, were placed in the thin glass bulb and connected electrically to the second calomel electrode by means of a loose mass of similar crumbs. Satisfactory voltage readings corresponding closely to those on suspensions (1 soil:1 water) of the same soil were obtained, provided the soil crumbs were moist enough to wet the glass membrane and establish an appreciable area of intimate contact between soil and glass. For soils below the "sticky point," where there was no such contact, the voltage readings, although constant and reproducible, resulted in pH values between 5.6 and 6.2, irrespective of the reaction of corresponding soil suspensions.

These values must therefore be taken to represent the pH value of a thin moisture film in equilibrium with the carbon dioxide content of the air and with the glass but not in equilibrium with the soil moisture. Similar results have been obtained by Kahler and Floyd Deeds(10), working in buffer solutions when only parts of the glass film were in contact with the solution.

Table I illustrates these results for a series of air-dried soils moistened with a spray of water and worked up into crumbs.

Table I. *pH values by glass electrode on soil crumbs.*

	Moisture as percentage of dry soil			
	10	20	50	100
Millstone Grit soil	5.3	5.3	4.1	4.1
Chernozem	5.7	5.8	6.0	6.1
Rothamsted soil	5.5	6.2	7.5	7.7

Most soils behave as the Millstone Grit soil in Table I. At a moisture content of about 30 per cent. the *pH* value changed rapidly; in this case it fell but in other soils it rose sharply.

A few soils showed a slower change over a wider range of moisture. Two of these are illustrated in Table I. In the highly granulated chernozem there was no sharp point of wetting with increasing moisture content, and in the Rothamsted soil the small fragments of calcium carbonate present may have come into contact with the glass membrane before the mass of the soil crumbs.

From the fact that the glass electrode gives satisfactory results for untreated field soils, moist enough to wet the glass membrane, it should not be concluded that the glass electrode possesses special merits for such work. On the contrary, the quinhydrone electrode may be used at lower moisture contents than the glass electrode, since to establish contact it is necessary to press the mixture of crumbs and quinhydrone on to the electrode; such pressure is not possible with the thin glass membrane.

Table II gives comparisons of glass and quinhydrone electrode measurements on crumbs, and glass electrode measurements on the corresponding soil suspensions for a soil treated with varying amounts of calcium hydroxide, and subsequently air dried and remoistened below the "sticky point." The quinhydrone electrode gives substantially correct but the glass electrode gives erroneous results in the dry crumbs.

Table II. *pH values by glass and quinhydrone electrodes on soil crumbs and suspensions.*

	Ca(OH) ₂ added as mg. eq. %			
	0	1	5	10
Quinhydrone measurements on crumbs	4.4	5.0	—	7.0
Glass electrode measurements on crumbs	5.5	5.5	5.5	6.2
Glass electrode measurements on suspension	4.5	5.1	6.0	7.4

32 *The Use of the Glass Electrode in Soil Reaction*

For measurements in soil suspensions the glass electrode provides a useful supplement to older methods with no special merits in cases where the quinhydrone method is applicable. It can, however, be used in solutions of much higher pH than the quinhydrone electrode and has the advantage for alkaline soils that no disturbance arises through removal of carbon dioxide, as in the hydrogen-electrode method. It is particularly suitable for measurements on highly oxidising or reducing systems and may therefore be used satisfactorily in soils with active oxides of manganese or iron, or in intensely reducing soils, such as are obtained by waterlogging in the presence of fermentable organic matter. Finally, it may be used in soils treated with hydrogen peroxide, sodium hypobromite, or similar materials.

OXIDATION-REDUCTION POTENTIALS.

Since the metallic electrodes used in oxidation-reduction measurements are readily polarised in all but the simplest systems, and since the potentials in complex systems must be considered in relation to the pH value or hydrogen-electrode potential, there is a definite advantage in using the glass electrode for such measurements. Its high resistance ensures that minimal currents shall pass through the system and the absence of added material simplifies the reaction measurements. The oxidation-reduction potentials, however, have proved to be easier to measure than to interpret, and the following results are recorded to indicate the complexity of the phenomena and to offer a warning against the increasingly common practice of drawing deductions from a few isolated measurements, often given without pH values.

There is a close formal analogy between the oxidation-reduction potential, measured at a polished platinum electrode in a "poised" system with oxidant and reductant in reversible equilibrium, and the potential of an electrode in equilibrium with both hydrogen gas and a "buffered" system containing a weak acid (or base) and its salt in reversible equilibrium. The potential varies with the ratio of reactants responsible for "poising" or "buffering." It is to be expected that in biological systems the active oxidants or reductants should themselves behave as weak acids or bases and the hydrogen-ion concentration must therefore be used in interpreting the oxidation-reduction equilibrium. In only a few simple cases has it been possible to work out the relationship of the intensity factor or measured potential to the quantity factor or amounts of oxidant and reductant. In the quinhydrone system the oxidation-reduction potential is so closely related to the hydrogen

potential that the measurement of this oxidation-reduction potential has become a standard method for the determination of pH values. If complex systems like soils contain material analogous to quinhydrone, it is obvious that no oxidation-reduction potential can be of any interest unless made at a measured pH value. Clark(4) and his co-workers have studied a great number of organic systems with the object of finding a range of oxidation-reduction indicators but only few of them provide simple relationships. There is little prospect at present of developing for many systems such theoretical relationships as hold for quinhydrone.

The empirical approach has been more successful and useful results have been obtained in biological systems. Thus in standardised media the form of the potential-time curve can serve to characterise groups of micro-organisms or cellular processes, *e.g.* denitrification.

Empirical methods have given valuable results in soil reaction work in spite of the fact that the exact nature of the acidic or basic materials present was unknown, and it might therefore be expected that a similar method could be used in oxidation-reduction measurements. There is, however, the essential difference that though it is possible by addition of alkali or acid to alter the pH value of the soil or other system and so study the resulting titration curve, there is no such simple method of altering in a reversible manner the oxidation-reduction potential of a soil or other complex biological system. The pH titration curves of soil are determined by the inorganic and organic colloidal fractions of the soil, both of which are relatively stable in nature and amount. If the oxidation-reduction potentials reflected the state of oxidation of the greater part of the iron and manganese compounds or of the organic matter of the soil, they would be of value in studies of soil formation and in connection with drainage and irrigation problems, whilst if they are determined by small amounts of active compounds produced by micro-organisms they would be too variable to be of general value, and it would become necessary to standardise empirical methods which would make the microbiological activity reflect either the reserve of readily oxidisable material or the environmental conditions.

The fundamental and pioneering work on soil oxidation-reduction potentials was carried out by Gillespie(11) in 1920. He studied the changes in oxidation-reduction potentials with time when soils were waterlogged with and without readily decomposable organic matter in the form of carbohydrates, and the potential-time curve in bacterial cultures under controlled conditions. The latter section of his work has been developed greatly through the work by Clark and co-workers,

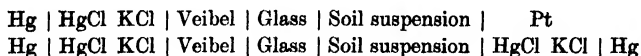
34 *The Use of the Glass Electrode in Soil Reaction*

Michaelis and others, but little has been added to the study of soil beyond establishing the fact that soils give definite and reproducible oxidation-reduction potentials under standardised conditions.

MEASUREMENTS OF OXIDATION-REDUCTION POTENTIALS IN SOIL-WATER SUSPENSIONS.

The underlying theory of oxidation-reduction potentials in general may be found in such text-books as those of Clark⁽⁴⁾ and Wurmser⁽¹²⁾.

If the glass electrode is used as a reference electrode, thus minimising polarisation and allowing simultaneous oxidation-reduction potential and *pH* measurements on the same soil suspension, the cells may be represented as:



The platinum electrodes (1 sq. cm.) were kept perfectly clean and bright. For each measurement two or more were used and the different electrodes usually agreed within 10 mv. The oxidation-reduction potentials (E_h) are given for the platinum electrode in the suspension relative to the normal hydrogen electrode as zero. They are thus positive for most oxidising systems and decrease with increasing reduction and with increasing *pH*.

The suspensions were made up from equal amounts of soil and water, but as the electrodes were always placed in the soil paste the precise soil-water ratio is of little importance. In general, constant and reproducible potentials were obtained within one or two hours and often more rapidly. Measurements in a nitrogen atmosphere gave the same results for the oxidation-reduction potentials as when air was present, a result which confirmed earlier measurements on soils by Herzner⁽¹³⁾. The measurements quoted later were obtained with air present.

The oxidation-reduction potentials of a wide range of English, European and tropical soils generally tended to follow the *pH* values.

A deep blue-grey waterlogged subsoil and some highly oxidising tropical soils showed less difference for similar *pH* values than did pairs of soils from adjacent experimental plots with widely differing reactions.

In Fig. 2 a few illustrative data of E_h and *pH* are given. Each section has, for reference, the E_h -*pH* relation for the quinhydrone and Fig. 2 *a* also gives portions of the hydrogen and the oxygen electrode potential lines. It will be seen that most soils fall close to the quinhydrone line and all on the more highly oxidising side of it. There was

no evidence that soils rich in manganese dioxide or ferric oxide gave especially high oxidation potentials, though they doubtless possessed great oxidising capacities. In every case high oxidation-reduction potentials of soils were associated with low pH values.

It is obvious that oxidation-reduction potentials measured under laboratory conditions must give only conventional values and that however useful these may be, they must differ from those under field conditions where the oxygen diffusion, water-level and the established microflora will affect the local oxidation-reduction level.

A waterlogged gley soil with characteristic iron mottling and dark stripes of manganese dioxide gave a moderately oxidising potential, even though it showed considerable amounts of ferrous iron when tested with acid and ferricyanide. The oxidation-reduction potential is thus no cri-

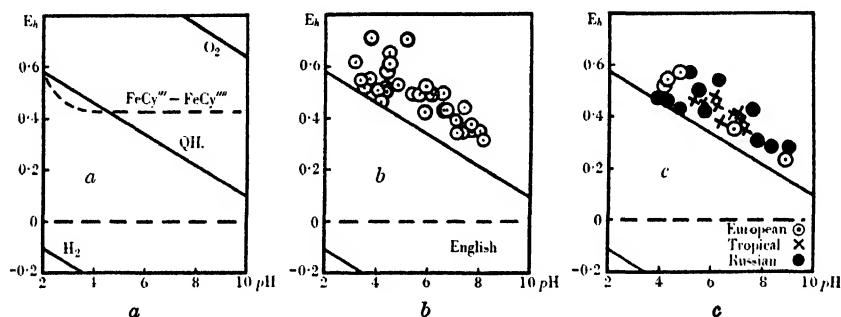


Fig. 2.

terion of waterlogging and formation of ferrous compounds. It would appear that with the alternation of reducing and oxidising conditions the products are laid down independently. Thus ferrous iron may form insoluble vivianite and higher oxides of iron and manganese may be deposited as gels which age rapidly to insoluble and inert forms. Such independent precipitation might explain the characteristic mottling and banding of gley soils. Clearly in such soils there is no approach to reversible equilibrium between the reduced and oxidised materials analogous to the equilibrium between bases and acids in kationic exchange.

Measurements on plots of varied manurial treatment show no appreciable effect of manures, which is in accordance with Herzner's finding for a large number of Austrian arable soils, but contrary to some observations by Remesov⁽¹⁴⁾ of marked effects on recently cultivated podsol soils rich in organic matter. This apparent contradiction will be discussed later. The changes in oxidation-reduction potentials with air drying are

36 *The Use of the Glass Electrode in Soil Reaction*

small for most soils. Appreciable differences were noticed only in soils with high contents of organic matter, the E_h becoming higher in some cases and lower in others, suggesting different forms of organic matter. Soils which have been air dried show no change in E_h over a long period.

Measurements of E_h changes when soils were treated with varying amounts of either HCl or $\text{Ca}(\text{OH})_2$ gave results of the type illustrated in Fig. 3, where the E_h -pH curves for some typical soils, as well as the quinhydrone line, are given. The majority of soils gave curves on the more oxidising side of the quinhydrone line and more or less parallel to it. From the similarity between the curves obtained from soils treated with

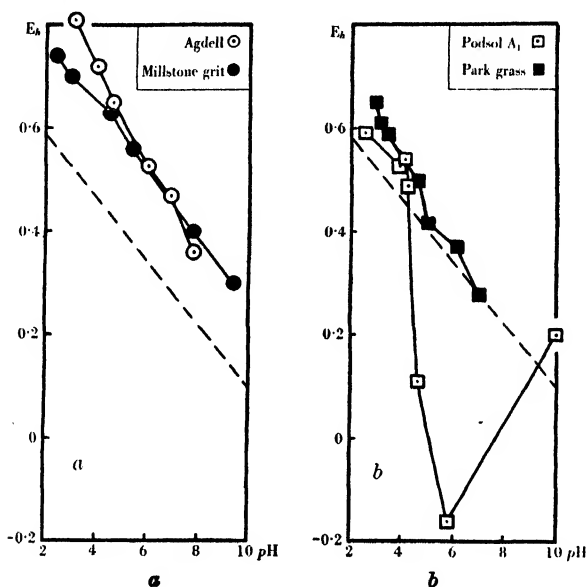


Fig. 3.

acid or base, and those from a wide range of soils at their natural pH values, with the quinhydrone line, it would appear that the system or systems governing the oxidation-reduction potentials were similar in nature to the quinhydrone system. Further, under normal conditions it seems likely that the factors responsible for the oxidation-reduction equilibrium are the reserves of oxidised and reduced materials rather than temporary occurrences of highly active materials.

Willis⁽¹⁵⁾ measured the oxidation-reduction potentials of an acid soil rich in organic matter after adding different amounts of lime. He concluded from the lower E_h values with the high additions of lime that in field liming the possibility of causing reducing conditions should be

remembered. This conclusion is, of course, quite unjustified, for all of his measurements are on systems more oxidising than quinhydrone and his low E_h values after liming merely express high pH values. The error illustrates again the necessity for considering the pH values in all E_h measurements. An instance of the effect of liming claimed, but not demonstrated, by Willis was provided by the titration curve in Fig. 3 *b* for the humus horizon of a sandy podsol which for small additions of base developed a marked reduction potential (E_h 0.2 at pH 6). The explanation of this fall in potential is given in the following section.

E_h CHANGES OF SOIL SUSPENSIONS ON LONG STANDING.

The measurements already recorded were made on soil suspensions which had stood for a few hours. On further standing in closed vessels, opened only for the measurements, there were but small changes in the potentials for most soils within 24–48 hours. If the soils were waterlogged for a considerable period most soils gave slightly decreasing E_h values. The highly oxidising soils poor in organic matter had in general greater capacity than the less oxidising ones for maintaining their potentials.

A very marked fall in E_h after waterlogging for one or two days was observed with some Russian chernozem soils. In order to ascertain how far this effect was characteristic of the chernozem soil type, measurements were made on a series of soil samples collected by Drs Crowther and Richardson during the 1930 International Soil Congress. The samples represent the major soil types of European Russia from the podsol soils of the north through the grey forest soils to the chernozems and the chestnut soils. They are arranged in Table III in order from north to south and are thus in approximate order of decreasing acidity and increasing alkalinity. Their fertility passes through a maximum in the chernozems. The last sample is from a great height in the Caucasus Mountains.

The E_h values of the freshly prepared soil suspensions showed only small and irregular differences according to soil type or horizon, but on standing for one or two days there was a clear distinction between a group of soils which gave low E_h values and the others which showed but small changes on standing. All of the A and B horizon samples from chernozem soils showed a marked fall in E_h , which in some cases led even to negative values, which however were not closely reproducible. The mountain meadow soil behaved in a similar way and the chestnut soils showed slight falls in E_h . The other soils and all of the C horizons gave

Table III. E_h and pH values of typical Russian soils.

Place	Soil type	Vegetation	Horizon	Depth cm.	E_h after			pH after		
					2 hours	24 hours	48 hours	2 hours	24 hours	48 hours
Lisino (near Leningrad)	Strongly podsolised on varve clay without gley formation	Mixed forest	A 2 B C	15-20 40-45 80-90	0.47 0.46 0.41	0.44 0.44 0.41	0.38 0.36 0.40	5.4 6.4 7.1	5.3 6.4 7.1	5.3 6.4 7.2
Starojilovo (Moscow District)	Dark grey forest soil	Cultivated for cen- turies after mixed forest	A 2 B 1 C 2	20-40 60-65 200-205	0.50 0.54 0.48	0.48 0.55 0.50	0.37 0.55 0.46	5.4 5.3 6.2	5.3 5.3 6.3	5.3 5.3 6.3
Starojilovo (Moscow District)	As above, in slight de- pression, highly de- graded	As above	A 2	15-20	0.51	0.54	0.54	5.4	5.6	5.6
Khrenovaya (Central Chernozem District)	Crusty chernozem- solonetz influenced by ground water	Dense grass regu- larly cut for hay	A B	0-10 10-20	0.48 0.49	0*	0.12 0.18	6.1 6.2	5.8 6.2	5.2 6.6
Voronesh	Chernozem (sample sup- plied by K. K. Gedroiz)	—	Surface	—	0.41 0.45	0*	0.25 0*	6.0 6.3	6.0 6.1	6.0 6.3
Izberdey	Deep chernozem	Cultivated	A 1 B 1 C	20-30 65-75 130-140	0.48 0.47 0.42	0*	0.25 0.44	6.2 6.4	6.1 6.4	6.2 6.9
Kharkov	Deep chernozem (southern form)	Cultivated	B C	40-50 100-120	0.41 0.41	0.35 0.41	0.32 0.42	6.8 7.6	7.7 7.9	7.7 7.7
Verbluid (North Caucasus)	Azov chernozem	Recently ploughed from shrub steppe	A	0-20	0.44	0.37	0.33	8.0	7.0	7.0
Gashun	Deep columnar solonetz of chestnut zone in very slight depression	Virgin steppe, medium grass	A B 1 C 1	0-8 10-20 50-60	0.45 0.37 0.40	0.37 0.27 0.45	0.31 0.20 0.43	6.9 7.5 7.5	6.7 7.6 7.8	6.7 7.5 7.8
Gashun	Chestnut	Virgin steppe, medium grass	A 1 B 1 C 1	0-12 25-45 65-95	0.35 0.39 0.42	0.18 0.33 0.40	0.34 0.33 0.40	7.3 8.0 8.0	7.3 7.5 8.1	7.2 7.4 7.9
Beketovka (near Stalingrad)	Chestnut soil slightly solonised	Virgin steppe	A 1 A 2 B 1	0-5 15-25 25-35	0.37 0.42 0.40	0.37 0.38 0.41	0.37 0.36 0.30	6.5 6.8 6.8	6.4 6.4 6.7	6.6 6.6 6.8
Georgian Military High- way (highest point)	Grey mountain meadow soil (altitude 2100 m.)	Much coarse grass	A 1 C	5-12 60-65	0.48 0.49	0.43 0.51	0.33 0.36	5.1 6.0	5.6 6.0	5.6 5.8

* The E_h values were poorly reproducible and varied irregularly from +0.2 to -0.2.

approximately constant values, with the exception of the C horizon of the mountain meadow soil, which yielded a considerable amount of ferrous iron after waterlogging. Even in chernozem soils, the fall of E_h can be completely inhibited by adding a trace of mercuric chloride or by treating the soil with dilute hydrogen peroxide. There can be no doubt that the fall in E_h is caused by microbiological activity in a system approaching anaerobic conditions.

Such falls in potential can be produced in any soil by addition of carbohydrates followed by waterlogging, but the form of the E_h -time curve for soils treated with glucose solution, in the manner originally studied by Gillespie, failed to give characteristic results for extreme kinds of soils. The E_h values reflect merely the intensity of the decomposition of carbohydrates by micro-organisms in the solution; the solid phase probably plays little part. Where, however, no carbohydrate is added to the soil, the E_h -time curves may be taken to show the presence or absence of readily oxidisable material in the original soil. Thus in the A and B horizons of the chernozem, in the mountain meadow, and in the humus horizon of a sandy podsol soil when limed in the laboratory, the marked fall in E_h indicates that readily oxidisable organic material is present. Most other soils, including an English fen soil, podsol soils and grey forest soils, do not contain such reserves of potential energy material.

In attempting to explain this grouping of soils it is convenient to consider first those soils which are sufficiently near neutrality to allow abundant bacterial activity when moisture and temperature conditions are suitable. The characteristic features of the chernozem belt of soils are low winter and high summer temperatures. The soil is moist for a short period in spring and early summer and under natural conditions a rapid growth of grass is followed by almost complete drying out. The plant roots and stubble decompose slowly on account of lack of moisture in autumn and of low temperature in winter. Organic matter accumulates, partly as material capable of being oxidised as soon as water supply and temperature allow, and partly as humus. In the laboratory experiments oxidation of the plant residues begins immediately, giving low E_h values comparable with those obtained when carbohydrates are added to other soils. In the drier chestnut soil region to the south of the chernozem belt the total plant growth is much less, and in the wetter north decomposition proceeds over much longer periods and little organic matter accumulates, except where some other condition, such as acidity, waterlogging, or low temperature, intervenes to restrict oxidation in the

soil. It is noteworthy that chernozems which have been under cultivation for centuries also show by low E_h values that readily oxidisable organic material is present. The residues of cultivated crops naturally behave as those of the native flora under the extreme climatic conditions and remain unoxidised until the spring. Evidence that the total organic matter content is not responsible for the fall in E_h on waterlogging is given by English fen soils which behave as normal English mineral soils. Fen soils which were drained centuries ago are not now accumulating organic matter; on the other hand they are slowly losing under cultivation the vast reserves of humified material built up when they were swamps. Fresh plant residues must now be decomposed rapidly even under normal winter conditions.

Under conditions of extreme acidity, as in the humus layers of podsol soils, readily oxidisable organic matter may remain and accumulate. Fig. 3 *b* shows that on neutralising such a soil in the laboratory intense reduction may result from the anaerobic decomposition of this material. Remesov found similar results on podsol soils recently limed in the field. On the other hand, some acid humus horizons, as *e.g.* on certain plots of the Rothamsted Park Grass experiments, do not show intense reduction even after neutralisation, as is shown in Fig. 3 *b*. Presumably in such cases only very inert forms of organic matter are accumulating.

The conditions determining the production and decomposition of organic matter in the soil may thus be used to interpret the main changes in oxidation and reduction on waterlogging, but the potential measurements do not appear to add much to what may be deduced from the current theories of soil formation. All the E_h values presented here are measurements of oxidation-reduction intensities. The difficulty of measuring "poising" capacities in complex biological systems becomes still greater when a third phase, the solid, is introduced by the soil particles. It has not been possible to find a suitably "poised" system which would allow true equilibrium. Systems like ferro-ferricyanide proved to have complex reactions with the soil, and potential measurements in slightly oxidising solutions such as dilute hydrogen peroxide were complicated by changes in *pH* values.

SUMMARY.

1. The glass electrode with an electrometer triode valve as amplifier gives accurate *pH* measurements on soil suspensions and on soil crumbs moist enough to wet the glass. It has the advantages that it may be used in highly oxidising or reducing systems and in alkaline soil, but it

has little merit over the quinhydrone electrode where this is known to be reliable.

2. The glass electrode forms a satisfactory reference electrode in oxidation-reduction potential measurements, as it allows both E_h and pH measurements without alteration to the system, whilst its high resistance minimises polarisation.

3. Oxidation-reduction potentials of soils depend so closely on the pH value of the soils that they should not be considered separately. For constant pH values highly contrasted soil types may give similar oxidation-reduction potentials.

4. After waterlogging in the laboratory for one or two days, there is a marked fall in potential for soils known from the conditions of their formation to contain organic matter capable of rapid decomposition as soon as moisture, temperature and soil reaction become favourable. In the main soil zones of European Russia, this change on waterlogging reaches its maximum in the chernozem belt.

In conclusion the author wishes to acknowledge her indebtedness to Dr E. M. Crowther, Head of the Chemical Department, for his valuable help throughout the work and in the discussion of data.

REFERENCES.

- (1) Report of the Committee on Soil Reaction Measurements. *Soil Research* (1930), **2**, 77.
- (2) HEINTZE, S. G. and CROWTHER, E. M. *Trans. Second Commission, Int. Soc. Soil Sci. Budapest* (1929), A, 102.
- (3) CROWTHER, E. M. and HEINTZE, S. G. *Trans. Second Intern. Soil Congress, Leningrad* (1930), **2**, 1.
- (4) CLARK, W. M. *The Determination of Hydrogen Ions* (1928), Baltimore.
- (5) HABER, F. and KLEMENZIEWICZ. *Z. physikal. Chem.* (1909), **67**, 385.
- (6) KERRIDGE, P. M. T. *Biochem. J.* (1925), **19**, 611.
- (7) MACINNES, M. D. A. and DOLES, M. *J. Amer. Chem. Soc.* (1930), **52**, 29; *Ind. Eng. Chem. Anal. Ed.* (1929), **1**, 57.
- (8) DOLES, M. *J. Amer. Chem. Soc.* (1932), **53**, 4260, 931, **54**, 3095.
- (9) HARRISON, G. B. *J. Chem. Soc.* (1930), **38**, 1528.
- (10) KAHLER, H. and FLOYD DEEDS. *J. Amer. Chem. Soc.* (1931), **53**, 2998.
- (11) GILLESPIE, L. *Soil Sci.* (1920), **9**, 199.
- (12) WURMSER, R. *Oxydations et Réductions* (1930). Paris.
- (13) HERZNER, R. A. *Z. Pflanz. Ern. u. Düng.* (1930) A. **15**, 342, (1931) A. **18**, 279.
- (14) REMESOV, N. *Z. Pflanz. Ern. u. Düng.* (1930) A. **15**, 34.
- (15) WILLIS, L. *J. Amer. Agron.* (1932), **45**, 571.

(Received June 1st, 1933.)

Soil oxidation-reduction potentials and pH values

(Oxydations-Reduktions-Potentiale und pH des Bodens. —
Les potentiels d'oxydo-réduction et le pH du sol)

by

S. G. Heintze,

Chemistry Department, Rothamsted Experimental Station, Harpenden.

The state of oxidation or reduction has long been recognised as one of the most important soil characters. It is readily observed in the field and plays a prominent part in the description and classification of profiles, but its quantitative measurement has received surprisingly little attention. Since Gillespie's famous paper in 1920 (1), on the oxidation-reduction potentials of soils waterlogged in the laboratory there has been little further progress. It has often been assumed that certain pathological disturbances in plants, such as the "grey speck" disease of oats on soils deficient in available manganese and the chlorosis of fruit trees on calcareous soils, depend on the degree of oxidation in the soil and should be connected with oxidation-reduction potentials, but no quantitative data have yet been obtained to substantiate these claims. Those who have measured the potentials at bright platinum electrodes immersed in soil-water suspensions have assumed that the potentials obtained were analogous to those for reversible oxidation-reduction systems, such as ferrous-ferrie salts. The potentials relative to the normal hydrogen electrode are expressed as EH values and, until recently, they were often given for soils without taking into consideration the pH values of the same suspensions.

The danger of drawing conclusions from EH values alone was stressed by the author (2) in a recent paper in which it was shown that when the EH values were plotted against the pH values of the same

suspensions, the points for a large number of widely differing types of soil were grouped around a line roughly parallel to, but more oxidising than the EH—pH line for quinhydrone. Similarly when varying amounts of acid and base were added to a soil the EH—pH points generally fell close to the average line through the points for miscellaneous soils. Most soils show somewhat lower EH values after liming without however indicating anything beyond a mere increase in pH. A few soils were found by Remezov (3) and the writer (2) to give a very marked fall in EH value after liming within a range of pH values, which allowed the active anaerobic decomposition of reserves of readily oxidisable organic material. All soils give very low EH values on waterlogging in the presence of added carbohydrates. Some of the soluble products of the microbial metabolism probably form approximately reversible oxidation-reduction systems.

On the assumption that the EH values in normal soils also represented true oxidation-reduction equilibria it appeared necessary to conclude (2) that most soils under natural moisture conditions must contain substances akin to quinhydrone in order to account for the observed general relationship between EH and pH in freshly prepared suspensions. There is however a simpler explanation. Unless the concentrations of oxidant and reductant exceed a certain limit the platinum electrode does not measure the ratio of reductant to oxidant but acts as a platinum—platinum oxide electrode. Even a bright platinum electrode becomes coated with a thin oxide film in air and this is sufficient to cause the potential to depend on the hydroxyl ion activity or pH value of the solution surrounding it. The platinum—platinum oxide electrode cannot be used accurately, but its potentials have been measured and some progress made in studying the irreversible changes in the thin film of oxide (4).

Measurements were therefore made with standard buffer solutions under the same conditions as had been used for soils. The results were reproducible to about 20 mv., and the whole of the data fell near a smooth curve. For a given pH value the potential varied somewhat with the nature of the acid present, but it is known that both the platinum—platinum oxide electrode potentials and true oxidation-reduction potentials are influenced by the anions present (4, 5). The EH values of the buffers are plotted against pH (as crosses) in Fig. 1, which also contains nearly all of the available data for soil-water suspensions (as circles) from the papers by Heintze (2), Remezov (3), Herzner (6), and Brown (7). Since many of these soils were selected to give the widest possible range of type and location, and since the measurements were

made by several workers, the fact that the data are grouped around the EH—pH curve for buffers suggests that the EH values for most soil-water suspensions are essentially rough pH measurements made by a platinum—platinum oxide electrode.

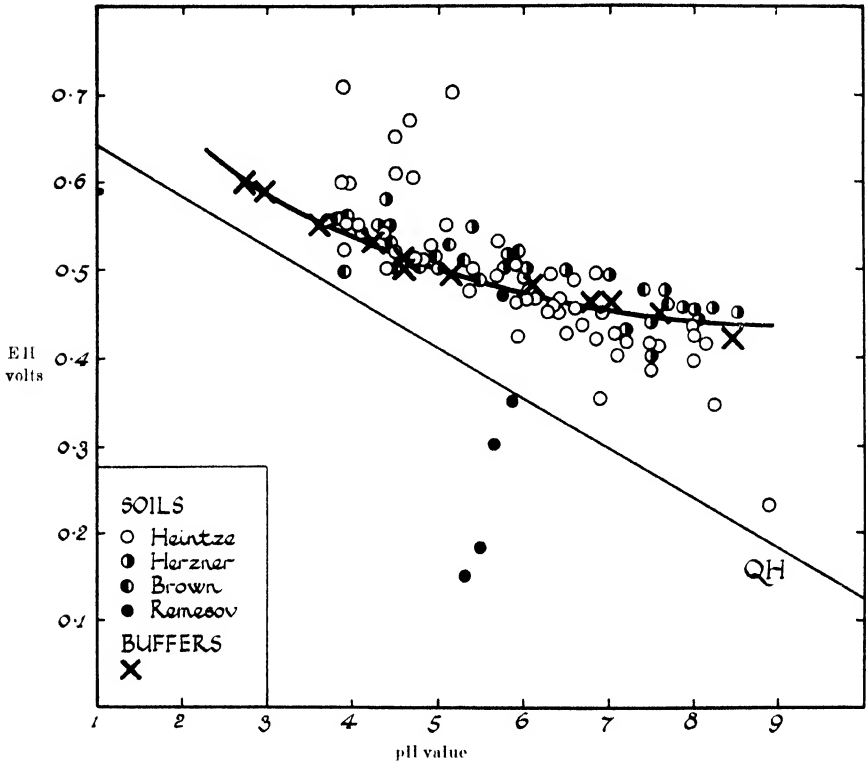


Figure 1

There are, however, as will be seen from Fig. 1, some soils which give EH values definitely above or below the general average EH—pH curve, and in these exceptional soils something more than mere pH value has been measured. Thus, some soils which are acid under natural conditions or are acidified in the laboratory, give high EH values. Under acid conditions the iron and manganese present in these soils may be dissolved in sufficient quantities to give approximately reversible oxidation-reduction systems. The very low EH values for a few soils may be due to highly reduced materials formed by anaerobic decomposition of organic matter. Soils rich in oxidisable organic matter

and naturally waterlogged, such as the gleypodsols measured by Remezov (3), probably resemble soil suspensions with added carbohydrate and the low potentials merely reflect the reduced conditions and oxygen shortage.

The oxidation-reduction potentials of suspensions of soil in dilute acid were used by Bradfield, Batjer and Oskamp (8) in an attempt to discriminate between soils under high- and low-yielding fruit trees in orchards. The EH values obtained when a fixed amount of soil was treated with a fixed amount of sulphuric acid were lower for the soil samples under low-yielding trees than those obtained from the soils under high-yielding trees. If however the EH values were corrected to comparable pH values, the correlation with yielding capacity was greatly weakened. From the data presented in the paper it is evident that the soils under low-yielding trees had higher initial pH values and higher buffer capacities, and probably contained calcium carbonate. They were also badly drained. With very few exceptions pH values determined before and after the sulphuric acid treatment would probably have sufficed to differentiate the soils. It seems undesirable therefore to replace accurately made and readily interpreted measurements by a single EH value which combines such dissimilar quantities as an initial pH, a buffer capacity and an oxidation-reduction potential at unknown or at least widely varying pH values. The empirical use of EH values for soils treated with a constant amount of acid may obscure vital differences between soils. It becomes particularly objectionable if for most soils the final measurements prove to be merely inaccurate pH values.

The platinum—platinum oxide electrode necessarily drifts as the thin film of oxide changes. To explain drifts in oxidation-reduction potentials, Wartenberg (9) had to assume that his soils contained some unknown substance which was oxidised only at the catalytically active surface of the electrode.

It is improbable that reversible oxidation-reduction equilibria are established in normal soils, or that EH values can be used for anything more than recognising acutely reducing conditions. No poisoning system is known in the soil analogous to the exchange complex which gives reversible equilibria for kationic exchange.

The author wishes to express her thanks to Dr. E. M. Crowther for criticism in the preparation of this paper.

References

1. Gillespie, L., Soil Sci. (1920), **9**, 199.
2. Heintze, S. G., J. Agric. Sci. (1934), **24**, 28.
3. Remezov, N., Z. Pflanz. Düng. (1930), **A, 15**, 34.
4. Hoar, S., Proc. Roy. Soc. (1933), **A, 142**, 628.
5. Michaelis, L. and Smythe, J. Biol. Chem. (1931), **94**, 329.
6. Herzner, R.A., Z. Pflanz. Düng. (1930), **A, 15**, 342; (1931), **A, 18**, 279.
7. Brown, L., Soil Sci. (1934), **87**, 65.
8. Bradfield, R., Batjer, L. P. and Oskamp, T., Cornell Univ. Bull. Nr. 592 (1934).
9. Wartenberg, H., Z. Pflanz. Ern. Düng. u. Bodenkde. (1935), **37**, 149.

AN EXAMINATION OF THE DEGTJAREFF METHOD FOR DETERMINING SOIL ORGANIC MATTER, AND A PROPOSED MODIFICATION OF THE CHROMIC ACID TITRATION METHOD

A. WALKLEY AND I. ARMSTRONG BLACK¹

Rothamsted Experimental Station, England

Received for publication June 1, 1933

Among the many methods of measuring the carbon content of soils two main groups may be recognized: those methods which determine the carbon dioxide produced by oxidation either in the wet or in the dry way, and those which in effect measure the reducing power of the soil by some oxidizing agent. Of the first type of method probably the only one which can be relied upon to give accurate results is the dry combustion method of Liebig or its modification by Dennstedt (3, 4, 5). The various types of wet combustion using chromic acid, though superior in accuracy to most of the methods based on reduction, frequently fail to give the complete oxidation usually claimed for them. The second or indirect type of method is based upon the assumption that carbon is the chief reducing agent present in soils and that any other substance exerting a minor effect will bear a constant proportion to the carbon. Any hydrogen atoms in the organic complex not fully oxidized already are likely to undergo oxidation along with the carbon, whereas nitrogen atoms, in taking up hydrogen to form ammonia, will act in the opposite way, so that one must assume that the proportions of carbon, oxidizable hydrogen, and reducible nitrogen are present in the organic matter of soils in about the same proportions. There is also the possibility of the presence of inorganic substances of an oxidizing or reducing nature, though probably these do not commonly occur.

Since indirect methods rarely give complete oxidation, we must make the further assumption that there is a definite fraction of organic matter common to many different kinds of soil which is readily attacked by oxidizing agents. In support of this there are the results of W. McLean (6), who has shown that 3 per cent hydrogen peroxide removes 85 per cent of the organic matter from a number of soils. Oxidations with sodium hypobromite and with chromic acid behave similarly.

Indirect methods may thus prove useful in the rapid examination of large numbers of soil samples for survey or advisory work when the necessary factor has been established by more exact determinations on typical ones. In the course of a comparative study by one of us on a number of such rapid methods,

¹ The authors wish to express their thanks to Dr. E. M. Crowther for a number of valuable suggestions and for his helpful criticism throughout.

the one proposed by Degtjareff (2) was carefully examined, for we could find no theoretical basis for its action.

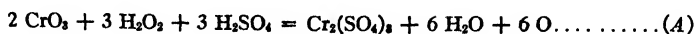
In the Degtjareff method 0.15 to 0.2 gm. finely ground soil is treated first with 10 or 15 cc. of 0.3 per cent hydrogen peroxide and then with an equal volume of a solution of 1.6 per cent chromic acid in concentrated sulfuric acid. Considerable heat is evolved. The mixture is shaken for a minute, washed into a beaker, and diluted to about 200 cc. Excess chromic acid is then titrated with ferrous ammonium sulfate in the presence of diphenylamine and from the amount of chromic acid reduced the carbon content of the soil is calculated.

The form of the titration, originally suggested by Schollenberger (8) was subsequently improved by him (9) by the addition of sodium fluoride, which makes the end point much sharper by preventing the buffering action of iron salts. We have used throughout Schollenberger's improved method of titration.

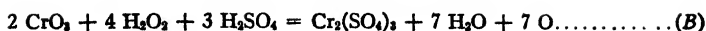
Since chromic acid and hydrogen peroxide react with mutual decomposition it is difficult to see what useful function the peroxide can perform other than that of providing water with which to generate heat when mixed with the strong sulfuric acid solution. It necessarily reduces the strength of the chromic acid, which is in excess, and if any organic matter is oxidized by the hydrogen peroxide or by nascent oxygen from its decomposition, the net result must be to reduce the apparent carbon content, since less chromic acid will then be used up.

With the short interval of contact between soil and the oxidizing agent it seemed most unlikely that oxidation of soil carbon could be complete. It thus appeared probable that some concealed error happened to cancel out the incompleteness of oxidation under the conditions used by Degtjareff. Preliminary trials soon showed that the apparent carbon content fell off rapidly when increasing amounts of soil were used in the determination. Since the work here reported was completed we have received a paper in which Tiurin (10) condemned the Degtjareff method and suggested that the reaction between hydrogen peroxide and chromic acid was incomplete but proceeded further in the presence than in the absence of soil. He produced, however, no direct evidence that any hydrogen peroxide remained in the presence of excess chromic acid, and it can easily be demonstrated that in such solutions no hydrogen peroxide does in fact remain. Ether extracts a deep blue perchromic acid from chromic acid solutions containing hydrogen peroxide, but fails to do so from the Degtjareff solutions (whether with or without soil).

Degtjareff and Tiurin assume that the reaction between hydrogen peroxide and chromic acid in strong sulfuric acid is



The nature of the corresponding reaction in *dilute* acid solutions was the subject of active investigations and discussions about 30 years ago (1, 7). Equation 4 was generally accepted, but an alternative one



was also proposed. The reactions in dilute solutions are naturally no guide to those in strong sulfuric acid solutions, but the possibility of altering the reaction by slight changes in conditions required examination. Our work indicates that soil acts catalytically and leads to the unusual situation that one reaction proceeds in the presence of soil and the other in the blank. The difference between the two parallel determinations with and without soil therefore fails to measure the carbon content of the soil, and it is an odd chance that the excess apparent carbon given by this change in the nature of the reaction should balance out the incompleteness of the actual attack on soil carbon for the conditions under which Degtjareff appears to have worked.

EXPERIMENTAL

The data quoted in this paper refer to determinations made on a standard series of seven representative British Soils selected by the Soil Analysis Subcommittee of the Agricultural Education Association for coöperative work. All samples were ground in either a porcelain or an agate mortar to pass a sieve with 100 meshes to the linear inch. (It may be mentioned that in any measurement of soil organic matter by reduction of an oxidizing agent such as chromic acid or sulfuric acid, very serious errors may be introduced, especially in sandy soils, if at any stage in the preparation of the sample an iron or steel mortar and pestle are used. Sufficient metallic iron is introduced into the sample to cause reduction comparable with that of a considerable amount of carbon in soil organic matter.)

The Dennstedt (3, 4, 5) method of combustion was used as the standard method for determining organic carbon. The soil was heated in a stream of oxygen, the products of oxidation passing over a heated sheet of platinum to ensure complete oxidation and then over lead peroxide to remove oxides of nitrogen and of sulfur and halogens. The carbon dioxide produced was determined gravimetrically. Inorganic carbon (as calcium carbonate) was present in only one (Rothamsted) of the seven soils. It was removed before the combustion by evaporating the soil to dryness on a water bath with excess of a solution of sulfurous acid. This removed carbonates and left a certain amount of sulfite which was oxidized to sulfur trioxide and absorbed by the lead peroxide in the Dennstedt combustion tube.

No sulfurous acid treatment was needed for the Dennstedt combustions on the six carbonate-free soils or for any of the experiments with chromic acid, for these all measure the reduction of the chromic acid and not the carbon dioxide produced in the course of the reduction.

The details as to strength and preparation of solutions used in the reduction methods are as described by Degtjareff and are given in the following:

- (a) Hydrogen peroxide—0.3 per cent.
- (b) Chromic acid—16 gm. CrO_3 is dissolved in a liter of conc. H_2SO_4 and the mixture heated to 165°C. for half an hour to stabilize it.

(c) Diphenylamine—0.5 gm. dissolved in 100 cc. conc. H_2SO_4 and 20 cc. water.

(d) Ferrous ammonium sulfate—0.2 *N*.

In each of the determinations by the reduction methods 0.15 gm. of 100-mesh soil was put into a 350-cc. flask into which was then pipetted 10 or 20 cc. of H_2O_2 , followed immediately by the same volume of chromic acid solution. The mixture after having been shaken for a minute was cooled and diluted to 150:200 cc. Next, 5 gm. sodium fluoride was added and when dissolved titrated with ferrous ammonium sulfate in the presence of 1 cc. of diphenylamine. Blank experiments were carried out under identical conditions.

Table 1 gives the results of the determinations by the Dennstedt method, by the Degtjareff method using two different amounts of reagents, and also by chromic acid with water instead of hydrogen peroxide.

The results of table 1 are plotted in figure 1. It is clear that the Degtjareff method gives excessively high values for low amounts of carbon and progres-

TABLE 1
Carbon determinations by the Dennstedt and Degtjareff methods

SOIL	WOBURN	HARPER ADAMS	ROTH- AMSTED	COCKLE PARK	BANGOR	CRAIB- STONE	INSCH
Per cent carbon by dry (Dennstedt) combustion.....	0.87	0.91	1.87	1.98	3.06	3.88	4.88
Per cent carbon by reduction method using:							
1. 20 cc. H_2O_2 + 20 cc. CrO_3	1.78	1.73	2.89	2.45	3.32	3.97	4.55
2. 10 cc. H_2O_2 + 10 cc. CrO_3	1.06	1.17	1.96	1.74	2.58	2.95	3.28
3. 20 cc. water + 20 cc. CrO_3	0.60	0.73	1.47	1.41	2.24	2.62	3.50

sively decreasing recoveries with increasing amounts of carbon. The apparent recovery reaches 100 per cent for 0.15 gm. of a soil containing about 4 per cent of carbon when 40 cc. of mixed reagents are used in the determination.

If x represents the percentage of carbon found by combustion and a the apparent percentage of carbon by the Degtjareff method using 0.15 gm. of soil with 20 cc. of hydrogen peroxide and 20 cc. of chromic acid solution, then the regression of a on x is found to be

$$a = 0.70 x + 1.22$$

Similarly if b is the apparent carbon content when 0.15 gm. of soil is used with 20 cc. of water and 20 cc. of chromic acid solution then

$$b = 0.69 x + 0.07$$

These two lines are plotted in figure 1. They show that the reduction of chromic acid corresponds to approximately 70 per cent of the carbon present in the soil together with a very large constant for the determinations with

hydrogen peroxide and with a trivial one for those with water instead of hydrogen peroxide. With 40 cc. of mixed reagents for 0.15 gm. of soil, the Degtjareff method would give an apparent carbon content of 1.2 per cent even if the soil were completely devoid of carbon.

The determinations with smaller amounts of hydrogen peroxide and chromic acid give results about midway between those just considered, when the soils have little carbon. For the soils rich in carbon the recovery falls off rapidly, since only very small amounts of chromic acid remained available for oxidation

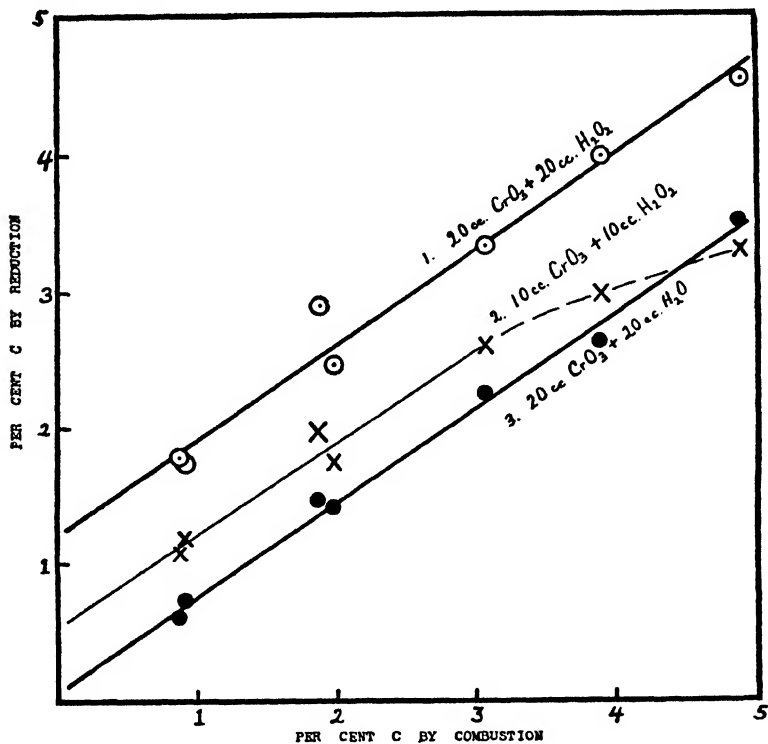


FIG. 1

after the joint reducing actions of the hydrogen peroxide and the large amount of carbon. With these smaller amounts of reagents a soil devoid of carbon would have an apparent carbon content of about 0.6 per cent.

It is clear from these results that the method as recommended by Degtjareff can have no value as an analytical method, and it becomes of interest to examine more closely the cause of its failure.

An attempt was therefore made to determine the quantitative relationships existing in the interaction of H_2O_2 and CrO_3 at the concentrations recommended by Degtjareff both in the presence and absence of soil.

This was done in the following manner:

- (a) 10 cc. of chromic acid solution was titrated with ferrous ammonium sulfate.
- (b) 10 cc. of chromic acid was mixed with 10 cc. of hydrogen peroxide and the excess chromic acid titrated as in (a).
- (c) The strength of the hydrogen peroxide was determined by adding to 10 cc. an excess of acidified ferrous ammonium sulfate and titrating back with potassium dichromate.

Table 2 gives the ferrous iron equivalents of the chromic acid (a), the hydrogen peroxide (c), and the excess chromic acid (b) in the mixture of chromic acid and hydrogen peroxide.

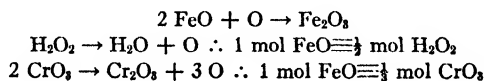
To obtain the molecular proportions reacting, these equivalents in terms of iron must be divided by 2 for the hydrogen peroxide, and by 3 for the chromic

TABLE 2

Ferrous iron equivalents of chromic acid, hydrogen peroxide, and excess chromic acid in chromic acid and hydrogen peroxide mixture

	TITRATION VALUE IN CC. 0.2 <i>N</i> FERROUS AMMONIUM SULFATE	TITRATION VALUE EXPRESSED AS MILLIMOLS FeO	EQUIVALENT IN MILLIMOLS
(a) 10 cc. CrO ₃	18.25	3.65	1.22 CrO ₃
(b) 10 cc. H ₂ O ₂ + 10 cc. CrO ₃	11.80	2.36	0.79 CrO ₃
(c) 10 cc. H ₂ O ₂	8.65	1.73	0.86 H ₂ O ₂

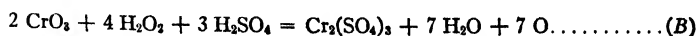
acid, as can be seen by considering the following equations, which represent the fundamental reactions occurring:



Then 0.86 mols H₂O₂ reacted with 1.22 - 0.79 = 0.43 mols CrO₃, i.e. 2 mols CrO₃ reacted with 4.0 mols H₂O₂. A subsequent independent determination gave the ratio as 2:3.8.

In these cases the peroxide was added to the chromic acid (which was in excess). When the reagents were mixed in the reverse order (as in the Degtjareff method) there was a temporary excess of peroxide in the early part of the reaction, which might possibly be expected to result in a ratio considerably less than 2 CrO₃:4H₂O₂. The ratio found, however, was 2:4.2.

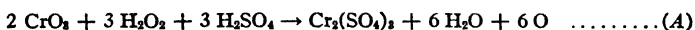
In the absence of soil therefore the reaction appears to be substantially that represented by the equation



When the hydrogen peroxide and the chromic acid were mixed in the presence of ignited soil it was found that the same amount of peroxide reduced more

chromic acid, as shown in table 3. Here 0.84 mols H_2O_2 reacted with $1.10 - 0.55 = 0.55$ mols CrO_3 , i.e. 2 mols CrO_3 reacted with 3.1 mols of H_2O_2 in the presence of ignited soil as contrasted with 4.0 mols of H_2O_2 in the absence of soil.

With ignited soil, then, the reaction may be represented by the equation



which was assumed by Degtjareff and Tiurin to hold both for the blank and for the actual determination on soil.

In a blank determination similar to that in table 2 and following equation *A*, the aforementioned amounts of reagents would react so that 1.10 millimols of $\text{CrO}_3 + 0.84$ millimols of H_2O_2 leave $1.10 - \frac{1}{2}(0.84) = 0.68$ millimols of CrO_3 instead of 0.55 millimols of CrO_3 . This difference, 0.13 millimols CrO_3 corresponds to an apparent carbon content of the ignited soil of 0.8 per cent and is

TABLE 3

*Ferrous iron equivalents of chromic acid, hydrogen peroxide, and chromic acid, hydrogen peroxide, and ignited soil mixtures**

	TITRATION VALUE IN CC. 0.2 <i>N</i> FERROUS AMMONIUM SULFATE	TITRATION VALUES EXPRESSED AS MILLIMOLS FeO	EQUIVALENT IN MILLIMOLS
(a) 10 cc. CrO_3	16.50	3.30	1.10 CrO_3
(b) 0.15 gm. ignited soil + 10 cc. H_2O_2 + 10 cc. CrO_3	8.25	1.65	0.55 CrO_3
(c) 10 cc. H_2O_2	8.45	1.69	0.84 H_2O_2

* The hydrogen peroxide and the chromic acid solutions used here were not those mentioned in table 2, but differed only slightly in concentration.

thus of the same order as the figure 0.6 per cent for the apparent carbon content of a soil devoid of carbon as obtained by extrapolation from the middle curve in figure 1.

The error caused by ignited soils appeared to be almost independent of the amount of soil taken.

Thus, a series with 0.07, 0.15, and 0.30 gm. of ignited soil with 20 cc. of mixed reagents gave excess chromic acid equal to 2.0, 2.1, and 2.2 cc. respectively of 0.2 *N* ferrous ammonium sulfate solution over and above the true blank. A little ferric oxide gave excess chromic acid equal to 1.7 cc.

The agreement between the values calculated from soils and those found directly for ignited soils is close enough to establish the disturbance of the reaction by soil, whereas the fluctuations in the actual value of the excess are sufficiently great to invalidate the Degtjareff method even if an attempt were made to improve it by allowing, first, for the essential difference between the reactions in the presence and the absence of soil, and second, for the incompleteness of the actual oxidation.

A PROPOSED MODIFICATION OF THE CHROMIC ACID TITRATION METHOD

From the parallelism of the lines for carbon recoveries in figure 1 it is clear that the oxidation of soil carbon proceeds just as far with chromic acid and water as with chromic acid and hydrogen peroxide and in addition that the line for the recovery with chromic acid and water passes so close to the origin that the apparent carbon contents are proportional to the true carbon contents. Although it is clearly impossible to base a rapid method on the use of chromic acid plus hydrogen peroxide, it should be possible to do so by the chromic acid plus water method provided it can be shown that the fraction of the total carbon recovered is reasonably constant for the groups of soil tested. Evidence for this possibility is afforded by Schollenberger's original proposal to determine carbon by treating a small amount of soil with 2 per cent potassium dichromate in strong sulfuric acid and heating to 175°C. in 90 seconds; by the work of Vassiliev (11), who used a slight modification of Schollenberger's method; and by Tiurin's method of treating the soil for 5 minutes with a boiling solution of chromic acid in 1:1 sulfuric acid and water. A considerable simplification in such methods may be effected by using the heat of reaction between sulfuric acid and water to avoid the necessity of external heating. This was the essential feature of Degtjareff's proposal though the principle was obscured and the method invalidated by using dilute hydrogen peroxide instead of water. There is a considerable advantage, however, in replacing the chromic acid-sulfuric acid solution, which is somewhat unstable, troublesome to make up, and difficult to measure out accurately, by aqueous potassium dichromate, which is free from these disadvantages. The necessary heating and acidity are obtained by adding sulfuric acid. Further work on the details of the method and on the significance of the readily oxidized fraction is in progress. In its present form the method is as follows. Finely divided soil, passing a 100-mesh sieve, is taken in amounts containing between 10 and 25 mgm. of carbon and placed in a 350-cc. conical flask. About 10 cc. of *N* potassium dichromate is then added from a burette, followed by 20 cc. of strong sulfuric acid, which is run in from an automatic pipette. The mixture is shaken for about 1 minute. After it has been cooled and diluted to about 150 cc., 5 gm. of sodium fluoride is added and the chromic acid remaining is titrated with 0.4 *N* ferrous ammonium sulfate, 1 cc. of $\frac{1}{2}$ per cent diphenylamine being used as indicator. Should the end-point be overshot, the mixture can be back-titrated with the potassium dichromate solution used as oxidant.

For a series of 20 British and foreign soils examined by this procedure about 76 per cent of the carbon was accounted for by the reduction of chromic acid. The actual recoveries ranged from 60 to 86 per cent, the standard error being ± 5.6 per cent. A correction factor of $100/76 = 1.32$ must thus be used (1 cc. *N* potassium dichromate equals $1.32 \times 3.0 = 4.0$ mgm. carbon).

Apart from the possible practical value of such an extremely rapid and simple titration method for approximate determinations of soil carbon, it is of

interest to have additional evidence that in soils of similar types the readily oxidizable organic matter forms an approximately constant fraction of the total organic carbon. In addition, the course of the oxidation of organic matter seems to be substantially the same for all of the British soils already discussed, since direct determinations of the products of oxidation showed that about 60 per cent of the total carbon went to carbon dioxide and about 3 per cent to carbon monoxide. This would suggest the almost complete oxidation of a group of compounds common to all of these soils.

SUMMARY

The chromic acid-hydrogen peroxide method of Degtjareff for the rapid determination of soil carbon is shown to give entirely fictitious results.

The hydrogen peroxide not only serves no useful purpose but introduces a fundamental error, since its reaction with chromic acid follows a different course in the determination with soil from that in the corresponding blank.

Two molecules of CrO_3 react with four molecules of H_2O_2 in the absence of soil but with three in the presence of soil or ignited soil. The gain in apparent carbon through this error approximately balances the incompleteness of oxidation for the conditions under which Degtjareff appears to have worked.

A new approximate method giving about 76 per cent recovery of carbon is proposed. Finely divided soil is treated with standard potassium dichromate and twice the volume of sulfuric acid added to raise the temperature; after being stirred for a minute the mixture is diluted and the excess dichromate titrated.

This procedure is more rapid than others so far proposed and it is believed that it may prove useful for comparative purposes where no very exact determination is required.

REFERENCES

- (1) BACH, A. 1902 Über das Verhalten der Chromsäure gegen Hydroperoxyd. *Ber. Deut. Chem. Gesell.* 35: 872.
- (2) DEGTJAREFF, W. TH. 1930 Determining soil organic matter by means of hydrogen peroxide and chromic acid. *Soil Sci.* 29: 239.
- (3) DENNSTEDT, M. 1919 Anleitung zur Vereinfachten Elementaranalyse. IV Auflage Otto Meissners, Berlin.
- (4) DENNSTEDT, M. 1924 Wahnschaffe u. Schucht. Wissenschaftliche Bodenuntersuchung, p. 76. Parey, Berlin.
- (5) DENNSTEDT, M. 1925 Abderhalden. Handbuch der biologischen Arbeitsmethoden, v. 1 (3): 483.
- (6) McLEAN, W. 1931 The effect of hydrogen peroxide on soil organic matter. *Jour. Agr. Sci.* 21: 251.
- (7) RIESENFELD, E. H. 1905-1908 Die Zersetzung der Chromsäure durch Wasserstoff Superoxyd. *Ber. Deut. Chem. Gesell.* (1905) 38: 2578; (1908) 41: 2826.
- (8) SCHOLLENBERGER, C. J. 1927 A rapid approximate method for determining soil organic matter. *Soil Sci.* 24: 65.
- (9) SCHOLLENBERGER, C. J. 1931 The determination of soil organic matter. *Soil Sci.* 31: 483.

- (10) TIURIN, I. V. 1931 A new modification of the volumetric method of determining soil organic matter by means of chromic acid. (Russian, with English summary.) *Pedology* 5-6: 36.
- (11) VASSILIEV, I. S. 1931 Zur Frage von den volumetrischen Bestimmungsmethoden des Humusgehaltes des Bodens. (Russian, with German summary.) *Pedology* 4: 34.

AN EXAMINATION OF METHODS FOR DETERMINING ORGANIC CARBON AND NITROGEN IN SOILS¹.

By ALLAN WALKLEY.

*(Chemistry Department, Rothamsted Experimental Station, Harpenden,
and the Waite Agricultural Research Institute, Adelaide.)*

(With One Text-figure.)

As a preliminary to the examination of a large series of soil samples from the plots of the Woburn Experimental Station, a critical study was made of a number of the current methods for determining organic carbon and nitrogen in soils. Special attention was given to relatively simple and rapid methods which are suitable for examining large numbers of closely comparable soils even though they give incomplete recoveries and need empirical standardisation. The modified Kjeldahl method of Robinson, McLean and Rice Williams⁽¹⁾ (subsequently referred to as the Bangor method) was finally adopted for the Woburn soils because carbon and nitrogen could conveniently be determined on the same soil sample. A modification of Schollenberger's rapid chromic acid titration method⁽²⁾ which was developed in conjunction with Armstrong Black and briefly described elsewhere⁽³⁾, was re-examined and the technique improved to give greater convenience and to overcome the errors due to the large amounts of chlorides in certain Australian soils.

THE DENNSTEDT DRY COMBUSTION METHOD FOR CARBON.

This method⁽⁴⁾ does not appear to have been widely used by British soil chemists, though it is well known on the Continent and has several advantages over the more usual dry combustion method originally due to Liebig. The combustion is conducted in oxygen which enters the combustion tube in two separate streams through concentric tubes, a long inner one, which is widened to admit the boat containing the sample, and a short outer one. The stream of oxygen passing through the inner tube and over the boat, sweeps along any unburnt gases and unites with the

¹ This paper is based on a portion of a "Thesis approved for the Degree of Doctor of Philosophy in the University of London", and some later work in Adelaide.

stream of oxygen from the shorter inlet tube. The streams meet near strips of platinum foil arranged to give a star-shaped cross-section and heated to low redness. Efficient oxidation on the platinum surface is an essential feature of the Dennstedt method. The combustion proceeds at a relatively low temperature, three ordinary burners replacing the usual array of burners or the powerful electrical heating units of the Liebig furnaces.

The only filling in the combustion tube is lead peroxide, which is held at about 320°C . and removes oxides of nitrogen and the halogens. Dennstedt advised the use of a mixture of lead peroxide and red lead, though Pregl⁽⁵⁾ used lead peroxide alone. This seems adequate, for some red lead is formed during the combustion. After considerable difficulty in obtaining lead peroxide free from organic matter, Merck's "special lead peroxide for Pregl method" was found to be satisfactory. Instead of using the lead peroxide in a boat open at one end, as recommended by Dennstedt, 30-50 gm. in the form of small granules were packed into a cylinder of copper gauze which filled the combustion tube. Dennstedt recommended that after removing the moisture by calcium chloride the carbon dioxide should be absorbed in a large soda-lime tube followed by a smaller one containing soda-lime and calcium chloride in separate compartments. In this work the large tube was found to be clumsy and was omitted. The smaller tube was filled with "sofnolite" and calcium chloride, and gave full absorption with a gas stream of 1-2 litres per hour. From the absorption tubes the gases passed through a wash-bottle containing a dilute solution of palladious chloride in which a black precipitate is formed by traces of carbon monoxide in the event of incomplete combustion.

With a 10×1 cm. boat and 100 mesh (0.13 mm.) soil yielding 0.1-0.2 gm. carbon dioxide the time for combustion was about 1 hour, but this might have been reduced by using a larger absorption tube. The analyses presented in Table III were made by the technique so far described. Some possible simplifications were subsequently investigated.

Owing to its dilution in a great body of inorganic material, the soil organic matter burns away quietly, and the operation does not require such careful attention as is needed for volatile or rapidly decomposing organic compounds. It seemed probable therefore that the apparatus might be simplified by omitting several refinements. The platinum star frequently glows brightly during the combustion of pure organic compounds, but it did not do so for soils, presumably because most of the oxidation proceeded at the surface of ferric oxide and other active in-

organic catalysts in the soil itself. A simplified apparatus with a single oxygen inlet and without a platinum star gave satisfactory results for several mineral soils. For a peat soil the result was about 2 per cent. too low and a trace of carbon monoxide was detected. This simplified technique has not been fully tested, but it is unlikely that any mineral soils would offer more difficulties than the peat.

On combustion amino- and imino-compounds liberate practically all of their nitrogen as gas, while substances with nitro- and azo-groups yield higher oxides of nitrogen (Pregl⁽⁵⁾, p. 28). It was found that about 15 per cent. of the soil nitrogen was recovered as nitrate in the lead peroxide filling, and since the amount of nitrogen in soil is commonly about one-tenth of the carbon, the error by neglecting the oxides of nitrogen would be only a small percentage of the amount of carbon. Analyses without lead peroxide filling confirmed the relative unimportance of this source of error. It is probable that the lead peroxide could be omitted in the combustion of many soils, but it is necessary to retain it where sulphurous acid has been used to remove carbonates and for soils containing sulphides or chlorides. Probably the most effective filling in a single-inlet combustion tube without a platinum star would be lead peroxide with some manganese dioxide.

THE BANGOR METHOD FOR CARBON AND NITROGEN.

The technique for the Bangor method of determining soil carbon by measuring the amount of the sulphur dioxide liberated in a Kjeldahl digestion was carefully standardised. The correction factors for carbon and nitrogen were found to vary with the temperature of digestion. In the original description of the method⁽¹⁾ it was stated that the liquid must boil vigorously, but considerable superheating is possible. In experiments in which the temperature of the boiling liquid was measured it was found that the percentage recovery of carbon from a Woburn soil increased from 84 per cent. at 337° C. to 92 per cent. at 355° C. For the present work and for the examination of the Woburn soils the conditions were closely standardised by giving a preliminary gentle heating for 30 min., until the contents of the flask reached the final colour, and then digesting for an hour with sufficient heating to cause the liquid to reflux in the neck of the flask. The recovery varied with the type of soil, but was reasonably constant and closely reproducible for similar soils analysed at different times. Thus, with the standardised heating the mean differences in percentage recoveries between duplicates performed at intervals of a week were 1.50 per cent. for carbon and 1.65 per cent. for nitrogen on a

group of twenty-six Woburn barley soils, as compared with 2.68 per cent. and 3.26 per cent. respectively for nineteen of the same soils without standardised heating. For seven Woburn soils the recovery of carbon with standardised heating varied between 92 and 95 per cent., and for fifteen soils from a rotation experiment in the Sudan Gezira between 81 and 87 per cent.

Two possible sources of error in grinding soils to pass the 100 mesh (0.13 mm.) sieve should be noted. A series of abnormally high results for some sandy soils by the Bangor method was traced to the presence of small particles of metal produced by abrasion during grinding in an iron end-runner mill. A trace of metallic iron will greatly increase the reducing power and apparent carbon content of a sandy soil in any method involving the reduction of an acid solution. Grinding by hand in a porcelain mortar was compared with grinding in a porcelain ball mill. Almost all clay soils examined gave similar results by both methods, but, contrary to expectation, sandy soils, which had lost their characteristically gritty feel by ball-mill grinding, gave lower results than by hand grinding. The apparent loss of organic matter proved to be due to dilution of the soil by material abraded from the mill. This was confirmed by the loss in weight of the mill.

The above experiments showed that extremely fine grinding was not essential for the Bangor method. In most soils good results were obtained with material ground to pass only a 1 mm. sieve; finer grinding is, however, often necessary for adequate sampling.

THE DETERMINATION OF NITROGEN IN HEAVY CLAY SOILS.

A heavy alkaline soil from the Sudan Gezira gave considerable increases in both carbon and nitrogen by the Bangor method as the result of fine grinding. In 1925 D. V. Bal⁽⁶⁾ showed that the Kjeldahl method gave considerably higher nitrogen contents in certain heavy Indian cotton soils when water was added before the sulphuric acid. At about the same time and independently, H. J. Page and the late G. C. Sawyer at Rothamsted observed the same effect for this Gezira soil. Recently other examples have been given^(7, 8). Bal suggested that the low results by the ordinary Kjeldahl procedure were due to the presence of some cementing substance soluble in dilute but not in concentrated sulphuric acid. The final residues are dark crumbs when concentrated acid is used directly, but after previous treatment with dilute acid, the final residues are finely divided and white. Sieving the Sudan soil to remove the nodules of calcium carbonate and manganese dioxide or previous leaching with

N/50 hydrochloric acid, followed by drying, had no effect on the recovery of nitrogen by treatment with concentrated acid. Very fine grinding without addition of water gave higher results than adding water before digesting the 1 mm. sample (Table I). In order to ascertain whether this

Table I. *Effect of fine grinding and water on nitrogen and carbon determinations in Gezira soil.*

	% nitrogen Kjeldahl method	% carbon Bangor method
1 mm. soil: Without water	0.0204	0.170
With water	0.0249	—
Ground in ball mill: Without water	0.0274	0.289
With water	0.0281	—

effect was general for heavy soils, a number of samples of widely differing types were digested for 1½ hours with 25 ml. concentrated sulphuric acid for the combined carbon and nitrogen determinations. Similar determinations were made, for nitrogen only, after previously allowing the soils to stand with 25 ml. of water for 15 min. (The addition of water interferes with carbon determinations by the Bangor method.) From the data in Table II it will be seen that only two soils, from Southern Rhodesia and the Sudan, showed marked differences between the "wet" and "dry" methods. Both of these soils were strongly alkaline. Since fine grinding overcomes the difficulty, it would appear that the error does not depend on the production of insoluble cements such as iron and aluminous sulphates, but on the failure of the crumbs in certain heavy alkaline soils to disperse in the non-polar sulphuric acid. For the Kjeldahl method the addition of water is not necessary for many heavy soils, but it is preferable, if only because the digestion proceeds more smoothly and with less

Table II. *Carbon and nitrogen determinations in heavy soils.*

Soil	Clay %	pH	% re- covery of carbon by Bangor method	% nitrogen by Kjeldahl method		
				"wet" a	"dry" b	$\frac{a-b}{a} \cdot 100$
Gloucester	41	7.1	84	0.548	0.542	1
Parleigh	32	7.0	96	0.233	0.234	0
Rothamsted	30	5.8	94	0.183	0.184	0
Cockle Park	30	5.6	93	0.150	0.155	-3
Rothamsted subsoil	—	4.2	—	0.101	0.102	-1
Mauritius	75	6.8	77	0.199	0.192	3
Southern Rhodesia (heavy vlei)	—	8.3	59	0.100	0.081	19
Malay	42	4.0	—	0.092	0.092	0
Southern Rhodesia (red soil)	—	6.6	77	0.064	0.065	-2
Tanganyika (lateritic clay)	—	5.2	76	0.054	0.053	2
Sudan Gezira	60	9.4	50	0.025	0.020	20

bumping. For heavy alkaline soils low in organic matter, it is advisable to grind the soil very finely, and also to add water. For the Bangor method it is essential that such soils should be ground very finely.

THE HYPOBROMITE METHOD FOR CARBON.

Sodium hypochlorite was used by Bornträger⁽⁹⁾ over thirty years ago for determining soil humus by titrating an alkaline extract until it became colourless. A known amount of Kasseler Brown was similarly treated and used as a basis of comparison. For rapid field survey work Lapicque and Barbé⁽¹⁰⁾ added hypochlorite directly to the soil and determined the excess iodometrically. Troell⁽¹¹⁾ showed that sodium hypobromite was effective in removing organic matter from soils. This reagent has the advantage that it is readily prepared by adding bromine to sodium hydroxide solution.

Experiments on the determination of soil organic carbon were made by shaking soil with hypobromite and allowing to stand overnight. Parallel experiments were conducted with Merck's Acidum Humicum and, later, with a soil of known carbon content as standard. An aliquot of the clear supernatant liquid was removed, treated with potassium iodide and hydrochloric acid and titrated with sodium thiosulphate in the presence of starch. Preliminary experiments showed that a considerable excess of sodium hypobromite was required and the solution finally adopted was prepared by adding 2.5 ml. bromine to 150 ml. *M* NaOH (giving 2 parts *M*/2 NaOBr to 1 part *M* NaOH). The change of hypobromite to bromate, thoroughly studied by Skrabal⁽¹²⁾, does not interfere with the titration, which depends on the total oxidising power of the solution. Titrations of the hypobromite alone by means of arsenious acid showed that in the above solutions only about 3 per cent. of the iodine titration value was due to bromate, and also that this amount was not increased in the presence of soil.

5 gm. of soil passing the 1 mm. sieve were treated with 100 ml. of the above solution, shaken a few times at intervals over 2 hours, left overnight and reshaken. When the soil had settled, 10 ml. of liquid were pipetted off, 10 ml. of 20 per cent. potassium iodide solution and 2 ml. of 6*N* hydrochloric acid added and the iodine titrated with *N*/2 thiosulphate. The results showed relatively poor recoveries, and the method was abandoned in favour of the dichromate method discussed below. Springer⁽¹³⁾ has suggested that the amount of decomposition of hypochlorite by unit soil organic matter might be used to classify soils according to the ease of oxidation of their organic matter.

THE DICHROMATE METHOD.

This modification of Schollenberger's rapid method (2) of determining organic carbon by oxidising it with chromic and sulphuric acids at 175° C. and titrating the residual chromic acid arose out of the examination of a proposed modification by Degtjareff (14). As already described by Walkley and Armstrong Black (3), the method consists in treating an amount of soil containing 10–25 mg. carbon and passing the 100 mesh (0.13 mm. sieve) with 10 ml. of $N K_2Cr_2O_7$ in a 350 ml. conical flask followed by 20 ml. of strong sulphuric acid from a quick-delivery automatic pipette. The heat evolved is sufficient to raise the temperature to about 120° C. The mixture is shaken for 1 min., cooled under the tap and diluted to about 150 ml. with water. 5 gm. of sodium fluoride are added, and as soon as this has dissolved the excess dichromate is titrated with 0.4 *N* ferrous ammonium sulphate, using 1 ml. of 0.5 per cent. diphenylamine as indicator. If the end-point is overshoot the mixture can be back-titrated with potassium dichromate solution used as oxidant. If more than 75 per cent. of the dichromate is reduced, the determination should be repeated with less soil.

The method is extremely rapid, taking only 10–15 mins., and should be useful in survey work where no great accuracy is required.

COMPARISON OF THE DENNSTEDT, BANGOR, DICHROMATE AND HYPOBROMITE METHODS FOR ORGANIC CARBON.

Table III gives the results of comparisons of the Dennstedt, Bangor, dichromate and hypobromite methods for a series of British and foreign soils. Eight of the soils were used in a co-operative study of the Bangor method by a Committee of the Agricultural Education Association, whose report (15) should be consulted for further details and recommendations. Some of the analyses in Table III were for single samples. The dry combustion method is the only one of the four which is capable of giving accurate results over a variety of soils. The variabilities of the recovery factors for the three other methods are shown by expressing the standard error per determination as a percentage of the mean percentage recovery. It will be seen that the hypobromite method gave much more irregular results than the Bangor or dichromate methods. The dichromate method was only slightly inferior to the Bangor method. It is clear that neither of these methods could be used for such a wide variety of soils as those in Table III, unless the appropriate recovery factors were determined for the main types of soil. Both methods may, however, be used

satisfactorily for groups of comparable soils with occasional determinations of the recovery factor. The Bangor method has the advantage of determining both carbon and nitrogen in the same sample, but the dichromate method is much cheaper and more rapid.

Table III. *Comparison of four methods for determining organic carbon in soils.*

Origin of soil	Description	% organic carbon by combustion	% recovery by		
			Bangor method	K ₂ Cr ₂ O ₇ method	NaOBr method
Great Britain:					
Arnish Moor*	Peat	45.8	101	86	—
Gloucester	Clay loam	5.43	84	74	62
Insch*	Loam	4.88	100	83	62
Craibstone*	Sandy loam	3.88	100	80	63
Bangor*	Clay loam	3.06	97	76	64
Purleigh	Clay loam	2.29	96	81	74
Cockle Park*	Clay	1.98	93	78	57
Rothamsted*	Clay loam	1.87	94	79	68
Harper Adams*	Light sand	0.91	99	72	—
Woburn*	Sandy loam	0.87	93	71	56
Abroad:					
U.S.S.R.	Chernozem	5.80	91	80	63
Swaziland	Heavy black soil	2.84	86	68	—
Mauritius	Heavy clay	2.18	77	78	71
Southern Rhodesia	Heavy black vlei	1.45	88	74	68
Malay	Clay loam	1.24	92	78	67
Swaziland	Red soil	1.19	89	76	—
Tanganyika	Heavy lateritic clay	0.63	76	75	60
South Australia	Sandy loam	0.40	90	74	50
Sudan Gezira	Heavy alkaline clay	0.38	85	60	—
Mean percentage recovery		—	90.4	75.8	64.7
Standard error per determination		—	7.7	5.6	8.3
Standard error per determination as percentage of mean recovery		—	8.6	7.4	12.8

* Soils used in co-operative work of Agricultural Education Association.

MODIFICATION OF THE DICHROMATE METHOD.

The dichromate method described above was subsequently re-examined at the Waite Institute, Adelaide, and some minor modifications were introduced to give more convenient working and to overcome difficulties due to chlorides. It was found that time could be saved without loss of accuracy (a) by using soil ground to pass the 0.5 mm. sieve instead of the 0.13 mm. (100 mesh) sieve, and (b) after digesting and shaking for about 1 min. by leaving the flask to stand on an asbestos sheet without further shaking for about 30 min. With large numbers of determinations it is convenient to carry through the digestions in order and then to proceed to the titrations when the first flask is cool. The speed and reliability of the method with these modifications may be illustrated for a series of analyses on fifteen red-brown earth soils from seven typical

South Australian profiles. Thirty determinations including sampling from 3-lb. lots of 2-mm. soil and grinding were carried through comfortably in one working day. The mean difference between duplicates was about 2 per cent. of the carbon content. In Fig. 1 dichromate results are plotted against dry combustion values, kindly supplied by Mr C. S. Piper. If the results had been corrected for the mean recovery factor of about 77 per cent. the errors of analysis would have been small by comparison with the inevitable errors of field sampling in most advisory and survey work.

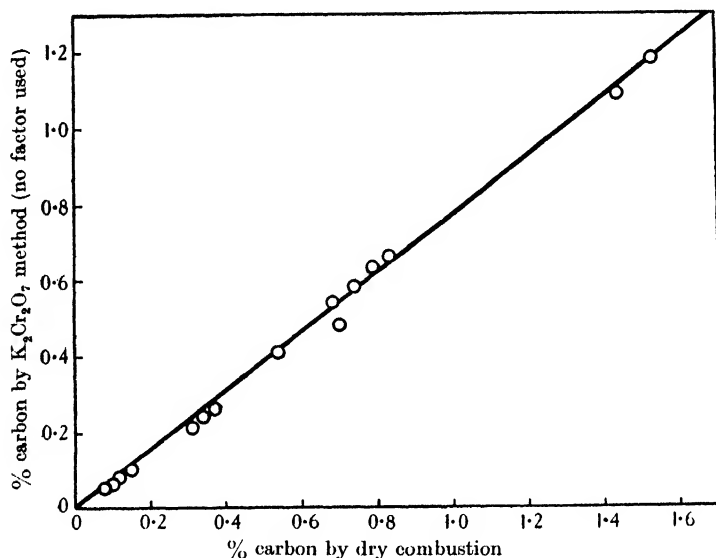


Fig. 1.

In the course of work for a co-operative investigation by a Committee of the Second Commission of the International Society of Soil Science, reported elsewhere (16), it was found from triplicate determinations on six soils that two other modifications of Schollenberger's method by Craig and Tiurin gave higher and more nearly constant recoveries between soils but less good agreement between replicates than the Walkley-Black method, with or without the above modifications. In the Craig and Tiurin modifications, as in the original Schollenberger method, the soil and chromic acid mixture is heated directly to much higher temperatures than are obtained through the heat of dilution of sulphuric acid in the Walkley-Black dichromate method. The simplicity and uniformity of treatment in the dichromate method thus compensates to some extent for

the incompleteness of the oxidation, and renders the method particularly suitable for comparing series of soils of similar types.

The violence of the reaction on adding the sulphuric acid varies according to the strength of the acid. Thus, with 98 and 96 per cent. acids the maximum temperatures reached were 120 and 115° C. Although the apparent carbon content by the weaker acid was 98 per cent. of that by the stronger, care should be taken to avoid acids below 96 per cent. sulphuric acid.

Soils containing considerable amounts of chloride gave unduly high results by the dichromate method. Dark fumes were liberated and condensed on the cooler parts of the flask as a heavy red liquid, presumably chromyl chloride. Some of this vaporised and the remainder was probably hydrolysed on dilution. Direct experiments with sodium chloride showed that the dichromate was reduced quantitatively ($1\text{C}=20=4\text{Cl}$). Since 12 parts of carbon are equivalent to 142 parts of chlorine, an approximate correction for the effects of chlorides may be made by assuming that the recovery of carbon is not influenced by the chloride, and subtracting 1/12th of the chlorine content of the soil from the apparent carbon content of the soil (or 1/9th of the chlorine content from the corrected carbon contents when the percentage recovery of carbon is about 75 per cent.). The results in Table IV for a Mallee soil with 0.12 per cent. chlorine show that this correction was satisfactory for the effects of added sodium chloride, until the Cl : C ratio approached 10.

Table IV. *Correction of dichromate titrations for the effects of chlorides.*

Cl %	Apparent C % by dichromate	Apparent C % minus 1/9 Cl %	C % by dry combustion
0.012	0.355	0.354	0.356
0.184	0.374	0.354	0.355
0.409	0.397	0.352	0.354
0.75	0.435	0.352	0.352
1.06	0.473	0.355	0.350
1.79	0.551	0.352	0.346
2.45	0.611	0.339	0.342
3.30	0.666	(0.299)	0.337

Where the chloride content of the soil is unknown its disturbing effect may be eliminated, at least in part, by precipitating the chloride as silver chloride. It was found that quite large amounts of silver sulphate (up to 150 gm. per litre) dissolve in concentrated sulphuric acid, though no quantitative data have been found in the literature. Sulphuric acid containing 100 gm. Ag_2SO_4 per litre gave an apparent recovery of 85 per cent. for a soil with over 4 per cent. Cl, as compared with 150 per cent. when no Ag_2SO_4 was used. The Ag_2SO_4 had only a small effect in non-

saline soils. Such large excesses of silver sulphate are probably unnecessary. If the Cl : C ratio does not exceed 1, then 25 gm. Ag_2SO_4 per litre H_2SO_4 should be sufficient to fix the chloride. For more saline soils the chloride content is a most important character of the soil and will generally be determined. The chloride may, of course, be removed by a preliminary washing with water.

It was found that nitrates up to 5 per cent. of the carbon content and carbonates up to at least 50 per cent. of the soil caused no disturbance. Manganese dioxide as an impure pyrolusite was added in amounts up to 3-4 times the carbon content without disturbance. It would appear that the high acid concentration prevents the oxidising action of the manganese dioxide.

$N \text{ FeSO}_4$ in approximately $N/2 \text{ H}_2\text{SO}_4$ was found to be more convenient than the more dilute ferrous ammonium sulphate previously recommended. Both solutions were kept under an atmosphere of hydrogen in stock bottles filled with self-adjusting zero burettes.

The method as at present modified is as follows:

Up to 10 gm. soil passing the 0.5 mm. sieve and preferably containing from 10 to 25 mg. carbon are weighed into a 500 ml. conical flask. 10 ml. $N \text{ K}_2\text{Cr}_2\text{O}_7$ are run in from a burette, followed by 20 ml. concentrated H_2SO_4 (not less than 96 per cent.) from an automatic pipette. The mixture is shaken for about 1 min. and left to stand for about 30 min. About 200 ml. water are added, followed by approximately 10 ml. of 85 per cent. H_3PO_4 and 1 ml. of 0.5 per cent. diphenylamine. The colour is not always purple on adding the indicator, but nearly always turns purple or blue just before the end-point is reached. The best procedure for titration is to run in the standard $N \text{ FeSO}_4$ solution till the colour is purple or blue, and then to continue the addition in 0.5 ml. lots until with little or no warning the colour flashes to green. 0.5 ml. of $N \text{ K}_2\text{Cr}_2\text{O}_7$ is added and the titration finished by adding the FeSO_4 solution drop by drop. It has always been found possible to determine the end-point to one drop. Often the blue colour does not reform after adding the 0.5 ml. $N \text{ K}_2\text{Cr}_2\text{O}_7$, but it soon reappears after adding a drop or two of FeSO_4 . If more than 8 ml. of the available 10 ml. of $\text{K}_2\text{Cr}_2\text{O}_7$ are reduced the determinations should be repeated with less soil. The percentage recovery varies somewhat with the type of soil and the details of technique, and should be determined by comparisons with dry combustions on similar soils. The author found mean recoveries of about 77 per cent., or a correction factor of 1.3 ($1 \text{ ml. } N \text{ K}_2\text{Cr}_2\text{O}_7 = 1.3 \times 3.0 = 3.9 \text{ mg. C.}$).

SUMMARY.

1. The details of the Dennstedt dry-combustion method for determining carbon in soils were described, and some simplifications suggested.

2. The Bangor modified Kjeldahl method for carbon and nitrogen in soils requires carefully standardised heating. Errors may arise from contamination of sandy soils by material abraded during grinding in iron or porcelain mills.

3. For many heavy soils the addition of water before the Kjeldahl digestion is convenient but not essential. For heavy alkaline soils with little organic matter it is advisable to grind the soil very finely and to add water.

4. The rapid dichromate titration method of Walkley and Black for soil carbon gave satisfactory approximate results. The details of the technique were improved and methods were devised for overcoming disturbances due to chlorides. The method should be useful in advisory and survey work in which the errors of soil sampling in the field are inevitably high.

The author wishes to express his thanks to Dr E. M. Crowther for his interest and assistance in this work, and to Mr C. S. Piper for supplying dry combustion data for the Australian soils.

REFERENCES.

- (1) ROBINSON, G. W., McLEAN, W. and RICE WILLIAMS. *J. agric. Sci.* (1929), **19**, 315.
- (2) SCHOLLENBERGER, C. J. *Soil Sci.* (1927), **24**, 65; (1931), **31**, 483.
- (3) WALKLEY, A. and ARMSTRONG BLACK, I. *Soil Sci.* (1934), **37**, 29.
- (4) DENNSTEDT, M. *Anleitung zur Vereinfachten Elementaranalyse* (1919), iv. Auflage.
- (5) PREGL, F. *Qualitative Organic Microanalysis* (1924).
- (6) BAL, D. V. *J. agric. Sci.* (1925), **15**, 454.
- (7) SRINIVASAN, A. *Ind. J. agric. Sci.* (1932), **2**, 5, 525.
- (8) SRINIVASAN, A. and SUBRAHMANYAN, V. *Ind. J. agric. Sci.* (1933), **4**, 646.
- (9) BORNTÄGER, H. *Z. anal. Chem.* (1900), **39**, 790.
- (10) LAPICQUE, L. and BARBÉ, E. *C.R. Acad. Sci., Paris* (1919), **168**, 118.
- (11) TROELL, E. *J. agric. Sci.* (1931), **21**, 476.
- (12) SKRABAL, A. *Mh. Chem.* (1911), **32**, 185.
- (13) SPRINGER, U. *Z. PflErnähr. Düng.* (1928), A **11**, 313; (1931), A **23**, 1.
- (14) DEGTJAREFF, W. T. *Soil Sci.* (1930), **29**, 239.
- (15) STEWART, R. and CROWTHER, E. M. *Agric. Prog.* (1934), **11**, 106.
- (16) CROWTHER, E. M. *Trans. Third Int. Cong. Soil Sci.* (1935), **1**, 114.

(Received February 25th, 1935.)

THE NUMBERS OF BACTERIAL CELLS IN FIELD SOILS, AS
ESTIMATED BY THE RATIO METHOD.

BY

H. G. THORNTON, D.Sc., AND P. H. H. GRAY, M.A.

631.476 576.809.34

*The Numbers of Bacterial Cells in Field Soils, as Estimated by the
Ratio Method*

By H. G. THORNTON, D.Sc., and P. H. H. GRAY, M.A., Bacteriology
Department, Rothamsted Experimental Station

(Communicated by Sir John Russell, F.R.S.—Received April 23, 1934)

1. *Introduction*

It has long been recognized that the method of counting bacteria in a soil sample by means of plate counts is valueless as an estimate of the total bacterial population, although such counts have undoubted value in comparing two or more samples. The numbers obtained by this method represent only a small fraction of the total population. This is due mainly to the fact that no single plating medium will enable all the diverse groups of soil bacteria to multiply and produce colonies. Indeed, many types will not grow on any of the media commonly used for plate counts, and it is very uncertain whether those which do so are of main importance in carrying out biochemical changes in the soil itself. The number of colonies which appear is probably reduced further by the fact that, in some soils, many of the bacteria occur in clumps which would not easily be broken up in the process of making the soil suspension. These limitations of the plate method make it very important that a technique should be developed for making accurate estimates of the total bacterial content of a soil sample from direct microscope counts.

The counting of bacteria in dried and stained films has become a recognized technique for milk (Breed, 1911). A comparison between plate and direct

microscope counts of milk samples was made by Breed and Stocking (1920) and more recently by Buice (1934) who found that the numbers obtained by two methods were of the same order. With milk, however, most, if not all, of the organisms counted were of types capable of growing upon the plating medium used. The application of direct counting methods to bacteria in a soil sample is greatly complicated by the presence of the soil particles. These introduce difficulties in staining the film, in obtaining an even distribution of bacteria over the film, and in estimating the mass of soil examined.

The difficulty in obtaining a differential stain for the bacteria in a soil film is due to the existence of particles of organic material which take up most of the stains commonly used for bacteria. Much of the soil organic matter consists of "humic acid" compounds which combine with the common basic dyes. Conn and Winogradsky found, however, that if an acid dye were employed, these bodies took up little or none of the stain, so that a differential stain for the micro-organisms could be obtained. Conn (1918) employed Bengal rose, but Winogradsky (1925) found that erythrosine gave a clearer stain. A modification of Winogradsky's staining method is employed in the technique here discussed.

By staining dried films of soil suspensions with these acid dyes, Conn, and especially Winogradsky, have made some very interesting qualitative observations on the appearance of bacteria in the soil itself thus opening upon a new field in soil microbiology. Koffmann (1928) developed a different and somewhat cumbersome technique in which a soil suspension, placed between a cover glass and slide, was fixed, stained, dehydrated, "cleared," and finally mounted in balsam by diffusion of the various liquids in from the edges of the cover glass. This method may have value for the qualitative study of delicate soil organisms that are destroyed in a dried film. It would seem too lengthy to form the basis of a quantitative method.

The preparation of dried and stained films of a soil suspension, however, is rapid and simple, and the use of such films for estimating the numbers of bacteria in soil naturally suggested itself. Indeed both Conn and Winogradsky have devised such methods.

Conn (1918) used a direct application of Breed's method already in use for counting bacteria in milk. One hundredth of a cubic centimetre of soil suspension of known dilution was spread over a square centimetre of microscope slide. The film was dried and stained and the bacteria in random microscope fields of known area were counted. Direct calculation from the mean count gave the numbers in the original film and suspension. An important factor

limiting the accuracy of this method must be the difficulty in obtaining an accurate sample of a suspension of soil particles only 0.01 cc in volume. Any error in the quantity of soil sampled will be multiplied by a large factor in calculating the numbers per gram. Winogradsky (1925) sought to estimate the quantity of soil in the film by weighing. Here again the error involved in weighing a soil film of perhaps 0.1 mg must introduce a large source of error in the final estimate of numbers per gram.

In both methods the calculation of the number of organisms per gram of soil involves an estimate not only of the small mass of soil in the film, but also of the number of bacteria in that film. Unfortunately this latter estimate is also liable to a grave source of error. It is based on the mean count of a number of random microscope fields and its reliability consequently depends on the assumption that the distribution of organisms over the film is random. An extensive series of counts which were made by Kühlmorgen-Hille (1928) enables this point to be tested. He made bacterial counts from 19 different soils using both Conn's and Winogradsky's methods and also the plate method. The two direct methods gave very different results. The actual counts obtained from individual microscope fields by each method are given and have enabled the present authors to test the agreement between such replicate counts within each sample by calculating the indices of dispersion according to the statistic

$$\chi^2 = S \frac{(x - \bar{x})^2}{\bar{x}},$$

where x = any individual count, \bar{x} = their mean and S stands for summation (Fisher, 1932). Where the distribution of the counts agrees with the expectation of random sampling, the value of χ^2 should approximate to the number of degrees of freedom. When using Conn's method, Kühlmorgen-Hille made counts of 40 parallel microscope fields from each of the 19 soils, thus obtaining a series of counts having $39 \times 19 = 741$ degrees of freedom. The values of χ^2 for the 19 sets total 1096.16, a value which is quite outside the expectations of random sampling. Winogradsky's technique involves the division of each soil sample into five fractions by sedimentation and centrifuging.* Kühlmorgen-Hille made counts from 24 random microscope fields from each of the five fractions of his 19 soil samples, thus providing 95 sets of counts, each having 23 degrees of freedom, from which to estimate the dispersion between replicate microscope fields. Of these, however, six sets gave such low counts, with many

* This division of the sample complicates the estimate of bacterial numbers owing to uncertainty as to the relative value to be assigned to counts from each fraction.

blank fields, that no valid estimation of χ^2 could be obtained from them. Table I shows the distribution of the values of χ^2 among the remaining 89 sets, together with the distribution to be expected in 89 sets of random samples. Column 2 shows for each value of χ^2 the corresponding magnitude of P, which is the percentage probability that a given value will be exceeded in a random sample. Here again there is a large excess of high values of χ^2 , indeed in 24 of the sets the values are larger than could occur once in a hundred random samples. One must conclude, in default of contrary evidence, that the bacteria in a dried film of soil are distributed so unevenly that *direct* counts from random

Table I— χ^2 indices from 89 sets of 24 replicate microscope field counts using Winogradsky's method (from Kühlmorgen-Hille's data).

χ^2 ($n = 23$)	P	Number of sets observed	Number of sets expected
		2	0.89
10.12	0.99	2	0.89
11.29	0.98	2	2.67
13.09	0.95	2	4.45
14.85	0.90	4	8.9
17.19	0.80	2	8.9
19.02	0.70	13	17.8
22.34	0.50	8	17.8
26.12	0.30	3	8.9
28.43	0.20	7	8.9
32.01	0.10	10	4.45
35.17	0.05	6	2.67
38.97	0.02	4	0.89
41.64	0.01	24	0.89

microscope fields do not provide valid samples of the whole film, and therefore do not provide reliable data from which to calculate the total bacterial content of that film. This conclusion, derived from Kühlmorgen-Hille's data, is confirmed by our results, given below.

There are thus two serious difficulties in estimating the bacterial numbers in a soil sample from the examination of stained films of that soil. The *first* is that of determining with sufficient accuracy the mass of soil in the film, the *second* that of estimating, from random microscope fields, the total number

of bacteria in the film, when these are not in fact distributed through it at random.* It seemed possible that these difficulties might be circumvented by the use of a *ratio* method similar to that devised by Wright (1912) in which a counted suspension of blood corpuscles is used to determine the density of a bacterial suspension mixed therewith. The principle of this method as applied to soil is as follows. A suspension of particles of distinctive colour is made up and the number of particles per cubic centimetre is determined. A known mass of soil is shaken up in a known volume of this suspension and films of the resulting mixture are prepared, dried, and stained. Counts are made of bacteria and of added particles in random microscope fields and the ratio of one to the other is determined. Since the absolute number of particles added per gram of soil is known, the number of bacteria is simply calculated. This method avoids the necessity of estimating the mass of soil in the film, since the calculation of bacterial numbers from the ratios depends upon the mixing of soil and particle suspensions in quantities large enough to measure with ease and accuracy. Moreover, the uneven distribution of bacteria over the dried film is mainly due to surface tension disturbances. So that, if coloured particles of the same size and density as bacteria are employed, it seemed probable that bacteria and added particles would be similarly acted on by surface tension forces, and that the ratio between them over different areas of the film would remain undisturbed during drying. One should thus obtain a more even distribution of ratios than of bacteria taken by themselves.

The properties required of coloured particles greatly restricts the range of substances that can be employed. They must be --

1. Insoluble in water.
2. Unaltered by drying.
3. Unaffected by the stain used for the bacteria.
4. Of a bright colour contrasting with this stain.
5. Transparent, so that the colour may show by transmitted light.
6. Of approximately the specific gravity and size of bacteria.

* The attempt to avoid the difficulties due to soil in the film was made by Vande Velde and Verbelen (1930). This method consists in shaking the soil with sterilized milk, plus a trace of formaldehyde, allowing it to settle and counting the bacteria in films of the supernatant fluid. The present authors know of no data enabling them to judge what relationship exists between the bacterial numbers in the supernatant milk and those in the original soil sample. A method somewhat similar in principle, in which counts are made from an alkaline soil suspension after centrifuging, has been evolved by Germanov (1932).

After trying many substances it was found that indigotin was the most satisfactory substance and that, in sufficiently small particles, it possessed all the above qualities.

2. Technique

The technique finally developed was as follows* :—

A—*Preparation of the Indigotin Suspension*—Half a gram of finely powdered indigotin is well shaken in 500 cc of sterile distilled water and the suspension is passed rapidly through coarse filter paper (*e.g.*, Whatman No. 4). The paper should be changed as soon as the pores show signs of becoming choked. The suspension is then counted on a hæmacytometer, 0.02 mm in depth. There should be about 500 million particles per cubic centimetre and their mean diameter should be about 1.5 μ . Unless the suspension is used at once, 0.2 gram of HgCl_2 per litre should be added to prevent bacterial growth. It is well to examine a stained smear of the suspension to ensure that it contains no stainable organisms.

Filtration does not ensure such uniformity in particle size as would be ideal. A great advantage would ensue if particles of more uniform size could be obtained. Satisfactory suspensions have been made by Dr. Hugh Nicol by chemical methods, but, hitherto, undesirable by-products have caused frothing in the presence of soil.

B—*Preparation and Staining of the Films*—The soil to be examined is passed through a 3 mm sieve and 5 grams are shaken with 25 cc of the counted indigotin suspension for 3 minutes.† The mixture is then diluted with 25 cc of sterile 0.01% agar and shaken for a further 3 minutes. The agar assists the subsequent adhesion of the film to the slide surface. About 5 cc of the mixture is quickly poured into a specimen tube for greater ease of manipulation. Five drops of about 2 mm diameter are placed on the surface of a carefully cleaned microscope slide by means of a mapping pen, the tube being shaken between the making of each drop. The drops are then left to dry. Such small drops dry rapidly and with a minimum of disturbance due to surface tension. Four or five replicate slides should be prepared from each soil sample. The slides are stained for 10 minutes in 5% erythrosine dissolved in 5% phenol, and restained for 10 minutes in saturated aqueous erythrosine washing in distilled water after each stain. The slides are then dried. The second staining intensifies the colouring of the bacteria. Other acid dyes can be used. Bengal rose,

* A note describing the method was published in 'Nature,' vol. 122, p. 400 (1928).

† Soils very poor in bacterial cells should be less diluted.

phloxine, cyanosine, and acid fuchsine have been tested but have not given better results than erythrosine.*

C—Method of Counting—The micro-organisms and indigo particles in a number of random microscope fields are counted under an oil immersion. It is best to insert in the eye piece a glass disc ruled with a square having 2 mm sides and to take only the area covered by this square as a field. Accuracy of counting is notably increased and eye fatigue reduced by the use of a binocular microscope. The number of fields to be counted depends upon the degree of accuracy required and upon the numbers of bacteria and indigo particles found per field (see below, p. 531). Where five drops on each of four slides are examined, it is usually sufficient to count 4 fields per drop so that the estimate of numbers is based on 80 fields. An important advantage of such microscope counts is that the preparations are permanent, so that the accuracy of a given estimate can be increased at any time by counting a greater number of fields. The number of organisms, o , per gram of soil is calculated according to the formula

$$o = 5y \cdot \frac{B}{I},$$

where y = the number of indigo particles per cubic centimetre of the suspension 5 cc of which were added per gram of soil, and B and I the total numbers respectively of organisms and indigo counted in the films. A typical series of counts is illustrated in Table II.

3—Tests of the Method

(a) *Agreement between Replicate Microscope Fields and Drops*—A serious source of error in other proposed microscope methods of counting soil organisms has been the uneven distribution of these organisms over the film. It was hoped that, in the present method, *ratios* of organisms to added particles might be more uniform over the film than was the distribution of the organisms taken by themselves. This can be tested by calculating the χ^2 indices of dispersion between replicate microscope fields. (For method of computing the ratio χ^2 , see Appendix.) These values have been calculated for nine series of counts from soils of three Barnfield plots. The results are shown in Table III where column A gives the number of degrees of freedom, n , for each count. Columns B and C show the χ^2 indices and the corresponding

* There is some confusion as to the terminology of the fluorescein group of dyes. The stain used is "erythrosine B," (tetra-iodo-fluorescein).

values of P for the counts of micro-organisms taken by themselves. Here, as with K  hlmoren-Hille's material, the micro-organisms are unevenly distributed over the films, the values of χ^2 being excessive in six of the nine sets, and in two of these being extremely high. Columns D and E show the χ^2 indices with their values of P for the *ratios* derived from the same replicate microscope fields. In only one set does the value of χ^2 exceed that expected

Table III— χ^2 indices of dispersion for ratios of organisms to indigo particles and for organisms alone in replicate microscope fields

Soil	A	B	C	D	E
	<i>n</i>	χ^2 of organisms alone	P	χ^2 of ratios	P
Barnfield plot 8-O—					
Sample 1	63	55.82	0.74	43.82	0.97
" 2	63	67.19	0.34	59.68	0.60
" 3	47	58.49	0.12	36.09	0.87
Barnfield plot 4-A—					
Sample 1	63	96.12	0.004	70.53	0.25
" 2	59	93.42	0.002	88.92	0.006
" 3	63	96.71	0.004	68.69	0.29
Barnfield plot 1-O—					
Sample 1	79	422.00	10 ⁻⁸⁰	84.53	0.32
" 2	63	86.66	0.025	79.30	0.08
" 3	63	143.81	10 ⁻⁷	43.26	0.96

in a random sample. Thus, although the micro-organisms were unevenly distributed over the films, the *ratios* of micro-organisms to indigo particles from the same microscope fields did not in general show excessive variability. This desirable result must be attributed to the surface tension forces acting similarly upon the micro-organisms and upon the indigo.

In examining a large mass of data it is more convenient to take the drop rather than the microscope field as the unit. A comparison of replicate drops also reveals any variability that may be introduced during the making of the drops. The values of χ^2 were computed for the ratios found in replicate drops from counts of 60 soil samples made by four workers over a period of some 4 years. The number of replicate drops was usually 15, but varied in different sets so that the χ^2 indices are not directly comparable. In order to collect the results into a simple diagram the values of P corresponding with each χ^2 index have been taken. Fig. 1 shows the distribution of these values, the broken line showing the distribution expected in random samples. The agreement between replicate drops is somewhat closer than would be expected from random sampling and in no set is the variability excessive. The placing of the drops on several microscope slides also enables any possible error due

to variation in staining to be detected, since the ratios from replicate slides should also agree within random sampling expectations. They have always been found to do this; an example is shown in Table IV. As regards the ratios, therefore, there is experimental justification for regarding the random microscope fields as valid samples of the whole drop, and the drop as a valid sample of the suspension. Since the ratios between replicate drops have been found to agree within random sampling, the variance between such drops will depend solely on the total numbers of organisms and indigo particles counted (see Appendix) and the percentage standard error can be calculated approximately from the formula

$$S \text{ per cent.} = 100 \sqrt{\frac{1}{B} + \frac{1}{I}} = s_1,$$

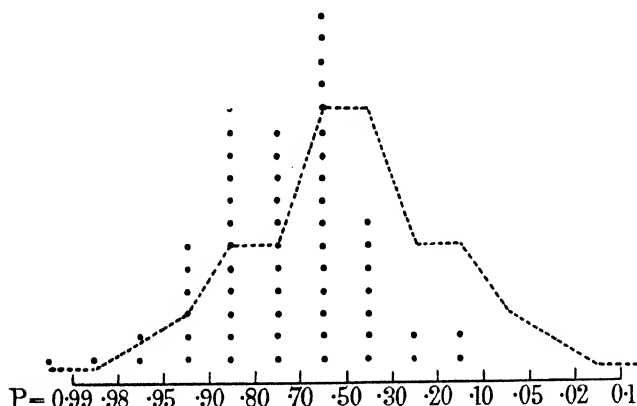


FIG. 1.—Distribution of the values of P for the χ^2 indices of dispersion between replicate drop ratios from counts made by four workers from 60 soil samples derived from various sources.

where B and I are the total numbers of organisms and indigo particles. In fig. 2 the percentage standard errors that should correspond to values of $\frac{1}{B} + \frac{1}{I}$ from 0.001 to 0.015 are shown by the curve, while the crosses show actual standard errors between replicate drop ratios calculated from 20 sets of data by the empirical method based on the natural logarithms of the ratios (see Appendix). This quick method of computing the expected standard errors, from the sum of the reciprocals of B and I affords a useful means of forecasting from a preliminary count from a small number of microscope fields, how many fields must be counted to reduce the standard error between drops to a desired percentage. Thus if a preliminary count from n_1 microscope fields gives a

standard error s_1 , the number of fields, n_2 , that must be counted to give a standard error s_2 can be obtained from the formula

$$n_2 = n_1 \left(\frac{s_1}{s_2} \right)^2,$$

such a calculation will often save counting an unnecessary number of fields. It should be noted that the standard error here involved is that between replicate drops and so does not take into account any variance due to the preparation of the films from the original samples. Any variance due to this cause, however, cannot be eliminated by counting a greater number of microscope fields.

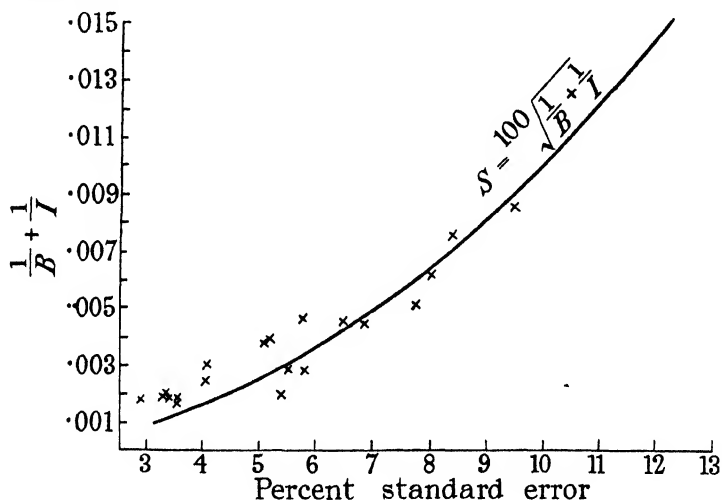


FIG. 2—Expected and observed standard errors of ratio counts plotted against $\frac{1}{B} + \frac{1}{I}$.

(b) *Consistency of Results*—In checking the reliability of the method it is further necessary to ascertain whether the personal factor due to the worker making the counts introduces any systematic error. Such an error might not affect the random distribution of the ratios found in replicate drops. It is also necessary to test the consistency of results obtainable when the method is applied to replicate fractions of a single sample of soil. Such a test covers possible errors involved in the whole process of preparing the films from the original soil. The following experiment was made to test these two points. A sample of soil from Plot 8A of Barnfield was passed through a 3 mm sieve and, after thorough mixing, was divided into four portions. Five slides, each with five drops, were prepared from each sample. Two workers made counts

Table IV—Counts of micro-organisms by two workers from four portions of a sample of soil from Barnfield

Sample	Counts by H. G. T. Five slides of five drops counted					Counts by P. H. H. G. Five slides of five drops counted					Mean ratios	Millions per gram of dry soil
	Organisms	Indigo	Ratios	χ^2 between slide ratios $n = 4$		Organisms	Indigo	Ratios	χ^2 between slide ratios $n = 4$			
A	1564	1024	1.527	2.9		1538	995	1.546	2.40	21.2	1.536	2758.2
B	1482	900	1.647	3.25		1498	897	1.670	3.30	12.4	1.658	2977.3
C	651	423	1.539	1.11		1298	841	1.543	1.13	21.0	1.542	2769.0
D	1420	815	1.742	4.87		1487	828	1.796	4.36	19.1	1.769	3147.8
Totals	5117	3162		12.13	$n = 16$	5821	3561		11.19	73.7		11652.3
Means			1.618					1.635	$n = 16$	$n = 100$	1.627	2913.075 ±93.08 (3.2%)

from different microscope fields from every drop. In the absence of a personal error their counts should agree as random samples. Table IV shows the numbers of organisms and of indigo particles counted and the resulting ratios obtained by each worker from each of the four portions. Column 10 shows the sums of the χ^2 indices of dispersion between the two workers' counts from individual drops of each portion. The two series of counts agreed within random sampling, no personal error being detected. Column 11 shows the mean ratios obtained from the two series of counts and the last column gives the numbers of organisms calculated per gram of dry soil, the moisture content varying slightly between the portions. The calculated numbers agreed with a standard error for the mean of 3.2% where that expected from random samples was 1.55%. The variance between portions is thus not wholly accounted for by random sampling. The extra variance was not due to the preparation of the films since replicate slides agree as random samples (see columns 5 and 9). It must thus have been present in the suspensions and probably represented a slight heterogeneity in the original soil portions.

A second experiment, with soil taken from the same plot some months later, was made to test the agreement between four portions of a single soil sample and gave the results shown in Table V. Here the standard error of the mean was 3.33%, that expected from random samples being 3.18%.

Table V—Counts of micro-organisms from four portions of a sample of soil from Barnfield

Sample	Organisms	Indigo	Ratios	χ^2 between drop ratios $n = 15$	Millions per gram of dry soil
A	477	424	1.125	13.72	2014
B	519	515	1.008	10.64	1815
C	459	419	1.109	14.02	1975
D	622	519	1.198	10.15	2135
Totals	2077	1877		48.53	7939
				$n = 60$	
Means			1.107		1984.75 ±66.05 (3.33%)

In the above experiments the same worker prepared the films from all the portions. It is important to ascertain whether any error is liable to be introduced by the worker's technique in preparing the films from the original soil. To test this, a sample of sifted garden soil was divided into three portions and slides were prepared and stained from each portion by a different worker. The microscope counts were all made by one worker with the results shown in Table VI. The very close agreement found shows that there was no difference due to workers' technique.

Table VI—Counts of micro-organisms from three portions of a soil sample, the preparations from each portion being made by a separate worker

Sample	Total counts of—		Ratios	χ^2 between drop ratios $n = 15$	Millions per gram
	Organisms	Indigo			
A (P. H. H. G.).....	1314	1693	0.776	4.86	1879
B (H. G. T.)	994	1264	0.786	14.98	1903
C (H. L. J.)	901	1168	0.771	13.4	1867
Totals	3209	4125		33.24	
				$n = 45$	
Means	1069.6	1375	0.778		1883
					± 10.58 (0.56%)

C—"Recovery" of Added Bacteria—The correct estimation by it of a previously known number of bacteria added to sterilized soil would seem to be the final test of the ratio method. This test has been made several times. The method was to count a suspension of a pure bacterial culture on a hæmocytometer, to add a known volume of this suspension to a known quantity of autoclaved soil and at once to prepare films from this soil and estimate bacterial numbers by the ratio method. The results of five such tests are summarized in Table VII.

Table VII—"Recovery" of counted suspensions of bacteria added to sterilized soil

Organisms added	Numbers added, millions per gram	Numbers found, millions per gram	Difference, millions per gram	Percentage difference
<i>E. coli</i>	8160	8220	+60	+0.74
<i>E. coli</i>	1160	1230	+70	+6.03
<i>M. piltonensis</i>	983.3	978.3	-5	-0.51
<i>R. meliloti</i>	880	847	-33	-3.75
<i>R. meliloti</i>	2080	2100	+20	+0.96

4—Numbers of Microbial Cells in Rothamsted Soil

The micro-organisms seen in stained films of Rothamsted soils consist for the most part of small rods and coccoid rods. Large rods found singly or in chains and *Azotobacter*-like cells are less common, but are fairly frequent in some samples. Cocci of various sizes are sometimes abundant, particularly in the Park Grass soils. Many of these are probably actinomycete spores, and the term bacteria, referring to direct counts, is used with this reservation. Fragments of actinomycete- and fungus-mycelium are numerically scarce as compared with bacterial cells. Cells which may be algæ or encysted protozoa are often seen, but the method is, of course, not adapted for preserving such

large cells sufficiently well to enable them to be identified with certainty. Their numbers are, moreover, too small for counts to be of much value. Trophic forms of flagellates have occasionally been identified. Diatoms also occur though in very small numbers.

Other observers have found that the soil bacteria occur mostly as clumps or colonies. In Rothamsted soil such clumps, sometimes imbedded in the soil colloids, are at first sight a striking feature, but the actual number of cells in these aggregates is small compared with that of isolated cells, which constitute more than 80% of the total numbers.

Table VIII shows the total numbers of micro-organisms per gram found by the ratio method in some Rothamsted soils, both arable and grass land, together with the results of plate counts of the same samples made on Thornton's medium (1922). The number of cells of micro-organisms in these soils varies from 1000 to 4000 millions per gram. Taking the average volume of such a cell as 1 cubic micron, 1000 million will occupy 1 cubic millimetre and weigh about 1 milligram. An acre of Rothamsted soil to a depth of 6 inches, if taken as weighing 1·5 million pounds will thus contain from 1500 to 6000 pounds of microbial substance having 300 to 1200 pounds of dry matter with 30 to 120 pounds of nitrogen.

Table VIII—Bacterial counts from Field Soils

Plot	Manuring	pH of soil	Total cell count millions per gram of soil	Plate count millions per gram of soil	Ratio of total count to plate count
Barnfield (arable ; continuous mangolds)					
1-O	Farmyard manure.....	7·6	3733	28·86	129·3
4-A	Complete minerals + ammonium sulphate	7·2	1766	15·1	117
8-O	No manure	8·0	1005	7·55	133·1
Park Grass (permanent meadow)					
13	Farmyard manure.....	4·6	2395	2·25	1064·4
11-1	Complete minerals + ammonium sulphate	3·8	2403	1·35	1780·0
12	No manure	5·6	3041	7·5	405·4

There is a striking discrepancy between estimates of bacterial numbers obtained by microscope counts and by plating (see Table VIII). This is particularly great in the grass plot soils, but in this instance the low plate counts may be due to the use of a plating medium originally devised for use with neutral soils and perhaps unsuitable for the growth of the predominant

organisms occurring in the acid grass plot soils. The plate counts represent an unknown fraction, probably small, of the total number of live bacteria in the soil whereas the microscope counts give the total number of live bacteria plus an unknown fraction of dead cells. It does not seem possible to determine the number of live bacteria in soil by means of the plate method because no single plating medium will support the growth of all the different groups of bacteria present, whereas if one attempts to overcome this difficulty by plating on several different media, there will be inevitable overlapping, for many types will grow on more than one medium. It can be shown that dead, or non-viable cells are included in the microscope count, since an appreciable number of cells can still be seen in stained films prepared from soil sterilized in the autoclave. Attempts to determine the number of live bacteria directly from microscope counts have therefore proved unsuccessful, although the authors have made numerous attempts to do this by various differential staining methods. It might be possible to derive an approximate estimate of the percentage of live cells included in the total count if the rate of disappearance of dead cells in the soil could be determined and were found to be approximately constant. Counts obtained by plating are derived from cells that are "live" in the sense of viable, *i.e.*, capable of multiplying on a suitable medium. There is no *a priori* reason why the number of viable cells should be more closely related, causally, to the biochemical changes in the soil than is the total number of cells, which may include many that are unable to multiply but are still able to produce chemical changes in their environment.

A survey of some of the barley plots on Hoos Field, Rothamsted, indeed, indicates a relationship between total numbers of micro-organisms and fertility. A series of soil samples were taken from 12 plots from this field on October 14, 1931, each sample being a mixture of four cores taken at random over the plot. From these samples the numbers of micro-organisms were estimated by the ratio method. Fig. 3 shows the average yield of straw for 60 to 76 years plotted against numbers of micro-organisms from 12 of these plots. There is a significant association, with a suggestion, however, that numbers of organisms are limited to about 3500 million per gram by some factor which did not similarly limit the growth of the crop.

Much more information concerning the behaviour of the total counts of micro-organisms in a field soil, will, however, be needed before their relationship to yield can be fully elucidated. For example, the sampling of the 12 plots on Hoos Field was carried out in about 1 hour, so that the time factor involved in this sampling may be important.

The plate count from soil samples taken at short intervals of time shows marked fluctuations (Thornton and Gray, 1930). It is necessary to discover how far these fluctuations also affect the total count before one can estimate the validity of counts from samples, taken at only one time. A series of samples was taken at 2-hourly intervals from a specially prepared plot of garden soil using the sampling technique described by Thornton and Gray (1930). On each occasion composite soil samples, each made by mixing six cores, were taken from the two halves of the plot and separate counts were made from each of the two samples by the ratio method. The numbers obtained are

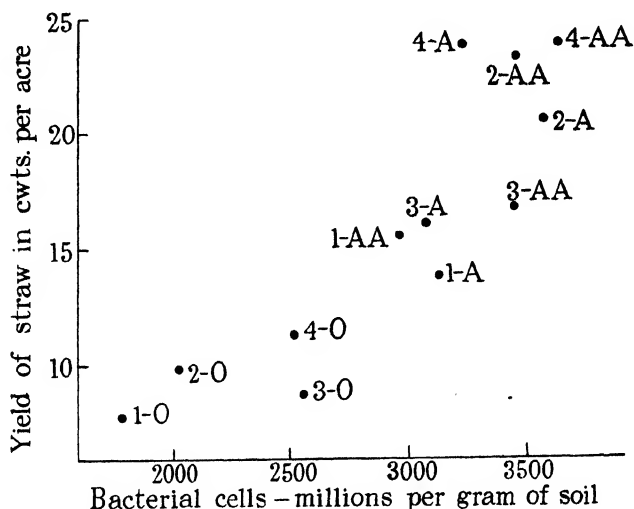


FIG. 3.—Bacterial numbers by the ratio method from Hoos Field plots compared with the yield of straw. The plot number is shown against each dot. (For manurial treatments see Rothamsted annual reports.)

shown in fig. 4. There was a marked fall in numbers during the period of sampling and this was statistically significant.*

The fact that the numbers of micro-organisms change not only in space over a field but also in time must greatly affect the technique of comparing the micro-population of two or more field soils. Thus if the taking of the samples to be compared be not strictly simultaneous, an error may be introduced owing to changes in the numbers of organisms during the period of sampling. Moreover, a single series of samples, even if taken simultaneously, may still

* The variance between simultaneous samples was 38,107 while that between mean counts at different times was 800,147. If these variances are compared by Fisher's method (1932) the value of z is 1.52 for which P is less than 0.01.

give misleading comparisons unless the fluctuations in numbers were to run parallel in all the soils compared. The comparison of the Hoos Field plots referred to above may be subject to error from both of these causes, though the present data as to fluctuations clearly provide no means of estimating the importance of such sources of error. Fluctuations in numbers of organisms in field soils must be investigated as a preliminary to any quantitative study of the soil population in the field, since the value of all such work is dependent on a knowledge of the variance due to fluctuations. The study and analysis of the fluctuations themselves must also be of fundamental importance to the extension of our knowledge of the factors that influence the activities of the soil population.

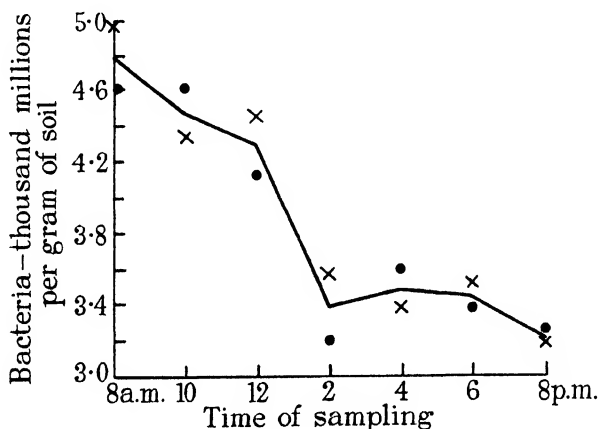


FIG. 4.—Microscope counts of bacteria from soil samples taken at 2-hourly intervals. The dots and crosses represent numbers found in the two half plots.

Acknowledgment

The authors gratefully acknowledge Mr. H. L. Jensen's assistance in making some of the preparations, the counts from which are shown in Table VI.

Summary

1.—There are two serious difficulties in estimating the bacterial numbers in a soil sample from the microscopic examination of stained films of that soil. The *first* is that of determining with sufficient accuracy the mass of soil in the film and the *second* that of estimating from random microscope fields the number of organisms in the film when these organisms are not distributed through it at random.

2—A technique is here described in which these difficulties are avoided, by determining, in random microscope fields from a parallel series of stained films, the *ratio* between the number of bacteria and the number of indigo particles, of which a counted suspension has previously been added to a given mass of soil. The bacterial numbers calculated from such ratios are, of course, independent of the mass of soil on the film.

3—It is found that the ratios obtained from parallel microscope fields are distributed at random, although counts of bacteria taken by themselves from the same fields are much less uniform.

4—The accuracy of the method has been tested in the following experiments :—

- (a) The bacterial numbers in four portions of a single soil sample agreed within a standard error of 3·3%.
- (b) The numbers found in films prepared by three workers from the same soil sample showed no significant differences.
- (c) The numbers found by two workers independently counting different microscope fields from the same films agreed as random samples.
- (d) Counted suspensions of bacteria added to sterilized soil were estimated with a standard error of 3·5%.

5—The bacterial numbers found in Rothamsted field soils by this method range from 1000 to 4000 millions per gram of soil.

6—Samples taken from some of the Hoos Field plots showed a relationship between the total bacterial numbers and the average yield of straw taken over a number of years.

7—Caution is at present necessary in discussing results obtained from samples taken on only a single occasion, since there is evidence of rapid changes in numbers of bacteria with time. Successive samples taken from garden soil showed significant changes in total bacterial numbers during the course of a day.

APPENDIX

By Professor R. A. FISHER, Sc.D., F.R.S.

It is important to avoid confusion between the two types of test for which χ^2 is used in this paper. If bacteria only are counted we have a single series of numbers representing the counts obtained from n' different fields, *e.g.*,

$$x_1, x_2, \dots, x_{n'}.$$

If the different fields contained equal quantities of soil, in which after thorough mixture the bacteria were dispersed at random, it is known (Fisher, 1932, sect. 16) that the number x should constitute a sample of n' from Poisson series, such that the probability of counting just x organisms is

$$e^{-m} \frac{m^x}{x!};$$

where m is the average number in all fields. When m is not unreasonably small, i.e., if it exceeds about 5 units, the homogeneity of the different fields may be easily tested by calculating what is known as the index of dispersion, defined as

$$\chi^2 = \frac{s(x - \bar{x})^2}{\bar{x}},$$

with $n' - 1$ degrees of freedom; for, on the hypothesis of homogeneity, this index will be distributed in a well-known distribution, independent of the value of m . Clearly when the values of x vary greatly χ^2 will be high, and since its distribution is known and readily available it is easy to see if its value is too high to admit of the hypothesis of homogeneity.

When both bacteria and indigo particles are counted, each field will provide two numbers, and we are concerned with the stability not of either number in itself, but of the ratio between them. This is equivalent to testing the proportionality of the entries in a $2 \times n'$ table of frequencies, for which the table of χ^2 may equally be used. If b and i stand for the numbers of bacteria and indigo particles counted in any field, and if B and I are corresponding totals for all fields, χ^2 may be written in the form (Fisher, 1932, sect. 21)

$$\frac{1}{BI} S \left\{ \frac{(bI - iB)^2}{b + i} \right\},$$

where the degrees of freedom are, as before, one less than the number of fields. Thus the same distribution may be used for testing the homogeneity of the ratios as for testing that of the absolute numbers.

Where homogeneity exists the importance of obtaining clear evidence of it is twofold. In the first place, the fact that the actual discrepancies between different fields, drops, or slides are of the same magnitude as those which would arise by pure chance in sampling homogeneous material affords a guarantee that the technique employed has attained the greatest possible precision subject only to the limitation imposed by the number of fields counted. In the second place, when the precision has been thus raised to the highest

possible level it depends only on the extent to which the material is counted, and can therefore be inferred from the total numbers. This is a considerable convenience in estimates of standard error, intended for use in tests of significance.

If p is the ratio $B/(I + B)$, then it is well known that, with homogeneous material, the variance of p is given by

$$V(p) = \frac{BI}{(I + B)^3};$$

from this, since I and B are both large, we may readily calculate the variance of the ratio B/I , and what is more important, that of its logarithm. For if $r = B/I$, then for small variations of p and r , dp and dr

$$\frac{dp}{p^2} = \frac{dr}{r^2},$$

whence

$$V(r) = \frac{r^4}{p^4} V(p) = \frac{(I + B)^4}{I^4} \cdot \frac{BI}{(I + B)^3} = \frac{B(I + B)}{I^3}.$$

Again if $Z = \log r$

$$dZ = \frac{1}{r} dr,$$

whence

$$V(Z) = \frac{1}{r^2} V(r) = \frac{I^2}{B^2} \cdot \frac{B(I + B)}{I^3} = \frac{I + B}{IB} = \frac{1}{I} + \frac{1}{B}.$$

To find the sampling variance of the natural logarithm of the ratio B/I , it is therefore only necessary to take the sum of the reciprocals of the total numbers of bacteria and indigo particles counted. This rapid and convenient test could not be relied on unless, as is shown by the χ^2 tests, practically all the existing variation is ascribable to purely random errors.

When this is not so, the values of Z derived from different drops will not agree so closely as is theoretically possible, and an empirical standard error based on actual deviations between values of Z for parallel drops should be used in tests of significance.

REFERENCES

- Breed, R. S. (1911). 'Zbl. Bakt.,' Abt. II, vol. 30, p. 337.
 Breed, R. S., and Stocking, W. A. (1920). 'New York Agric. Exp. Station Technical Bull.,' No. 75.
 Buice, W. A. (1934). 'Zbl. Bakt.,' Abt. II, vol. 89, p. 387.

- Conn, H. J. (1918). 'New York Agr. Exp. Station Technical Bull.,' No. 64.
- Fisher, R. A. (1932). "Statistical Methods for Research Workers" (Oliver and Boyd).
- Germanov, F. N. (1932). 'Proc. Second Int. Cong. Soil Sci., Commission III,' p. 239.
- Koffmann, M. (1928). 'Zbl. Bakt.,' Abt. II, vol. 75, p. 28.
- Kühlmorgan-Hille, G. (1928). 'Zbl. Bakt.,' vol. 74, p. 497.
- Thornton, H. G. (1922). 'Ann. Appl. Biol.,' vol. 9, p. 241.
- Thornton, H. G., and Gray, P. H. H. (1930). 'Proc. Roy. Soc.,' B, vol. 106, p. 399.
- Vande Velde, A. J. J., and Verbelen, A. (1930). 'C. R. Acad. Sci. Paris,' vol. 190, p. 977.
- Winogradsky, S. (1925). 'Ann. Inst. Pasteur,' vol. 39, p. 299 (see p. 342).
- Wright, A. E. (1912). "Handbook of the Technique of the Teat and Capillary Glass Tube" (Constable), p. 185.

REPRINTED FROM
THE JOURNAL OF EXPERIMENTAL BIOLOGY,
VOL. XII, No. 1, pp. 52-58, JANUARY 1935.
[*All rights reserved.*]

.

THE EFFECT OF BACTERIAL PRODUCTS ON AMOEBIC GROWTH

BY D. WARD CUTLER AND LETTICE M. CRUMP.

(Rothamsted Experimental Station.)

(Received 1st September, 1934.)

(With Four Text-figures.)

THE interrelationships that exist between various groups of Protozoa in nature and the bacteria which occur in the same environments are undoubtedly of more than academic interest. This is especially the case perhaps in soil, where both types of organisms are present in very large numbers, and where a marked degree of activity is evidenced by the continual fluctuation to which these numbers are subject. The food relationships of one member of the soil population, the amoeba *Hartmanella hyalina*, was described in a previous paper⁽²⁾, where it was shown that certain kinds of bacteria afforded a more efficient food supply than others as judged by the reproductive rate of the amoeba. *A priori* this might have been due to the fact that the bacteria giving a high reproductive rate to the amoebae were of greater food value than the others; or that the by-products formed by the less valuable bacterium in the course of growth were deleterious in their effects; but preliminary experiments indicated that for the species of bacteria concerned it was a true food value effect and not one caused by the presence of any by-products.

The type of problem is seen in the following inconclusive experiment where single amoebae (*Hartmanella hyalina*) from a pure culture with "YB"¹ bacteria were isolated into soil extract to which approximately equal numbers of bacteria of four kinds were added. The bacteria selected were "YB," "SE,"¹ *B. prodigiosus* and *B. fluorescens liquifaciens*. After 24 hours' growth the reproductive rates of the amoebae were very different in the four cases (Table I). The reproductive rate for any time is calculated from the formula $\frac{\log B - \log A}{\log 2}$: where *A* is the number at the beginning and *B* that at the end of the period.

Table I. *Average reproductive rate of Hartmanella hyalina, single-cell cultures, with four species of bacteria in 24 hours.*

	"YB"	"SE"	<i>B. prodigiosus</i>	<i>B. fluorescens liquifaciens</i>
Average reproductive rate	3.24	1.68	0.5	1.75
No. of cases	4	4	4	4

¹ For description of these bacteria see Cutler and Crump (1927, p. 157).

It is impossible to say whether the "YB" bacteria alone provided a really adequate food supply, or whether the other three types in varying degrees produced conditions inhibiting the reproduction of the amoebae. Since in all cases a certain number of "YB" bacteria are present, carried over with the single amoeba, it is probable that at least in the case of *B. prodigiosus* there was a definite check, other than lack of food, on the growth of the amoebae. On the other hand, further experiments have shown that by continuous subculturing it is possible to keep *Hartmanella* alive in pure culture with *B. prodigiosus*¹. If amoebae that have been grown with *B. prodigiosus* and "YB" respectively are isolated into soil extract containing on the one hand *B. prodigiosus* and on the other "YB," the results suggest that continuous growth with the former has produced a tolerance to the otherwise unsuitable bacterium (Table II).

Table II. Average reproductive rates of single-cell cultures of *Hartmanella hyalina* with *B. prodigiosus* and "YB" in 24 hours.

Subculture fed with "YB"	Parent grown with	
	<i>B. prodigiosus</i>	"YB"
" " <i>B. prodigiosus</i>	3.56	3.93
" " "	2.09	1.15

In the experiments so far quoted the bacterial numbers were not counted; but in all parallel experiments the initial numbers were the same and the reproductive rates are therefore comparable.

If growth products are responsible for the lowering of the reproductive rate of the amoeba a similar effect should be obtained by using a filtrate made from a culture of the appropriate bacterium. Previous work with the bacterium "SE" and *B. mycoides* showed that this only obtained when old cultures were used; further work with *B. prodigiosus* has given the same result (Table III). In this experiment

Table III. Average reproductive rate of *Hartmanella hyalina* in 48 hours in presence of filtrates from bacterial cultures.

	Parent <i>B. prodigiosus</i> Fed <i>B. prodigiosus</i>		Parent "YB" Fed "YB"	
	Filtrate "YB"	Filtrate <i>B. prodigiosus</i>	Filtrate "YB"	Filtrate <i>B. prodigiosus</i>
Average reproductive rate in 48 hours	5.25	5.19	7.89	7.45
No. of cases	5	5	5	6

amoebae from two pedigree strains of *Hartmanella*, one with *B. prodigiosus* and one with "YB," were isolated into soil extract containing 50 per cent. filtrate of each of the two species of bacteria prepared from young cultures. Each subculture so

¹ In an earlier paper (2) this was stated to be impossible; experiments in that case were carried out on nutrient agar.

made was fed with the type of bacterium to which it was accustomed. From the results it would appear that the effect of the filtrate was negligible compared with that produced by the direct feeding.

There is, however, the further possibility that though a filtrate from a growing culture gave no effect, yet one obtained from disintegrated bacterial cells would give a positive result. *B. mycoides* was chosen to test this point, the cells being thoroughly ground in a glass bacterial mill with hay infusion and the resulting suspension filtered. At this time a culture of *Hartmanella* was not available, but another common soil amoeba (*Naegleria gruberi*) was used. The active forms of these two species are almost indistinguishable, although the cysts are different, and the latter reproduces more slowly than the former. For example, with similar bacterial feeding the average reproductive rate in twenty-four hours for eight single-cell

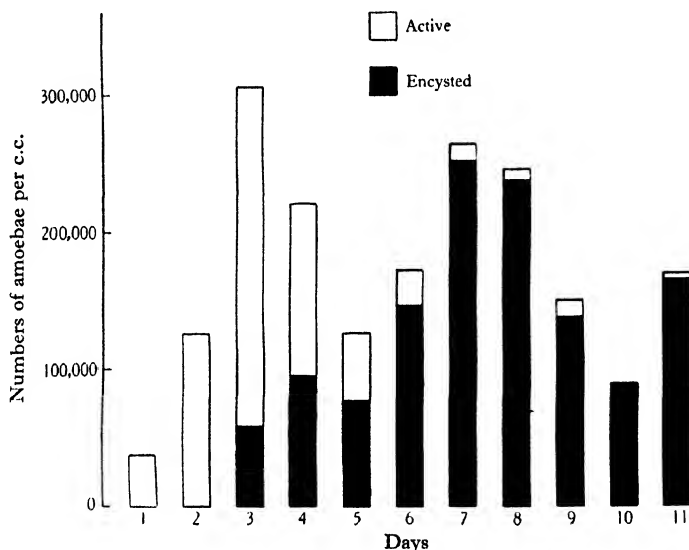


Fig. 1. Control culture in hay infusion.

cultures of *Naegleria* was 2.93, while that for *Hartmanella* was 6.63. On this occasion mass cultures instead of single cells were used, as the cultures were to be kept for some time so that daily observations could be made to determine the changes in the numbers of the population and the time of the onset of cyst formation.

In the control (Fig. 1) the end of the initial growth period is reached on the third day, and of the total population of 307,500 amoebae, 57,500 were encysted. The toxin culture (Fig. 2), however, was in comparison much less active and the initial peak was not only delayed by twenty-four hours but was much lower, the total number present being 140,000 of which all were encysted. After these periods the numbers in both cultures agree in falling, and in the toxin culture remain more or less constant to the end of the experiment, while with one exception 100 per cent. are in the form of cysts; whereas in the control, although the percentage of cysts

still remains high, there is definite evidence that reproduction is still taking place since the numbers are rising and falling.

As it was evident that the toxin was exercising an inhibiting influence this culture was centrifuged to collect the amoebae and the supernatant fluid was boiled for half an hour; to this material the amoebae were returned and the experiment continued (Fig. 3). Boiling had no appreciable effect.

The control culture was similarly treated and behaved in the same manner. The apparent discrepancy between the numbers at the end of Fig. 1 and at the beginning of Fig. 3 in this culture is due to the fact that the volume of the fluid was reduced and therefore the concentration of organisms per cubic centimetre was increased. After seven days the cultures were again centrifuged and boiled, and on this occasion the amoebae from each culture were divided and while half were returned to their own fluid (Fig. 3) the other half were put into sterilised hay infusion (Fig. 4). Again in the case of the original toxin solution boiling had no

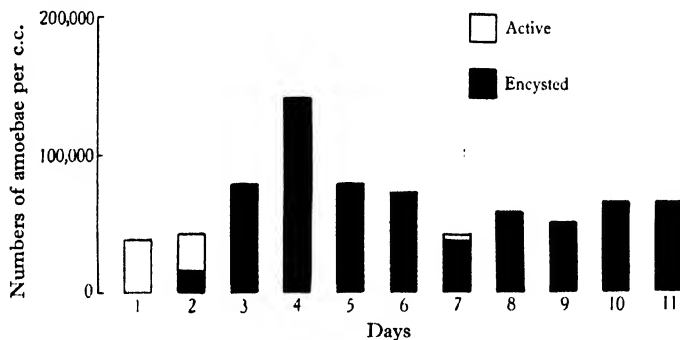


Fig. 2. Toxin culture in 50 per cent. hay infusion and 50 per cent. *B. mycoides* toxin.

effect, although on the twenty-second day there is a drop in the numbers, implying that a small amount of excystation had occurred followed by death since on the previous day there were 100 per cent. cysts.

The original control, which had also been boiled, gave much the same result, though there was an initial excystment, accompanied by slight reproduction, after which it behaved like the toxin culture, though to a greater extent, there being excystation followed by death.

The cysts from both cultures which had been put into new medium showed an entirely different picture as regards activity, for not only did considerable excystation occur, but this was accompanied by a reproductive rate of 0.94 in one case (control) and 2.59 in the other (toxin).

Judging from the results obtained from the treated culture the effect of the *B. mycoides* toxin was twofold, firstly it undoubtedly depressed the whole level of activity, and, secondly, it appears to have encouraged cyst formation. The question of the causation of cyst formation is very much debated; an idea that adverse external conditions such as lack of food, drought and toxic conditions are the sole

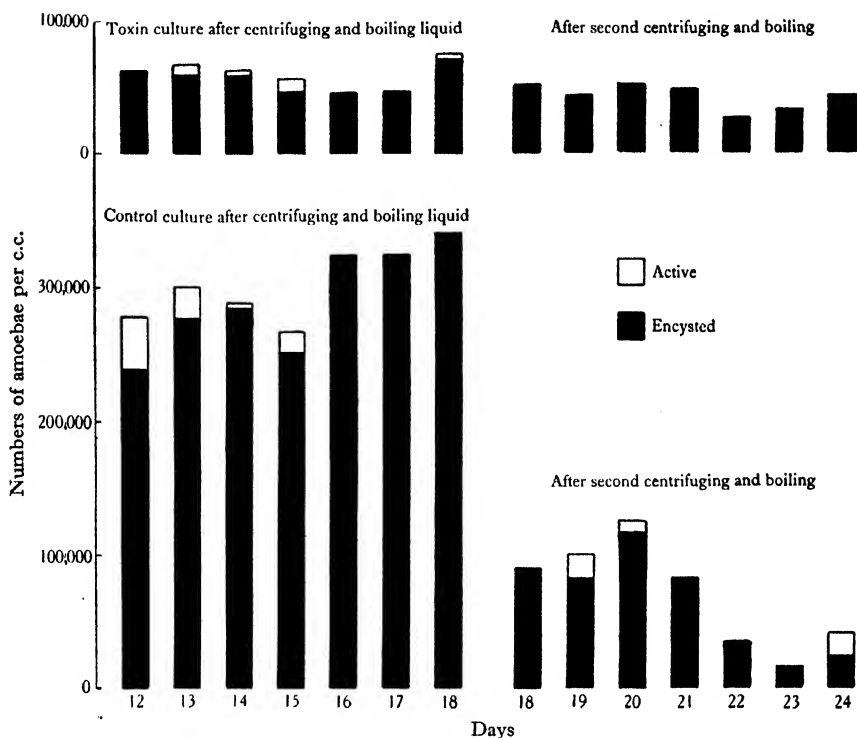


Fig. 3. Growth of amoebae in control and toxin cultures after centrifuging and boiling supernatant fluid and returning the organisms to the boiled liquid.

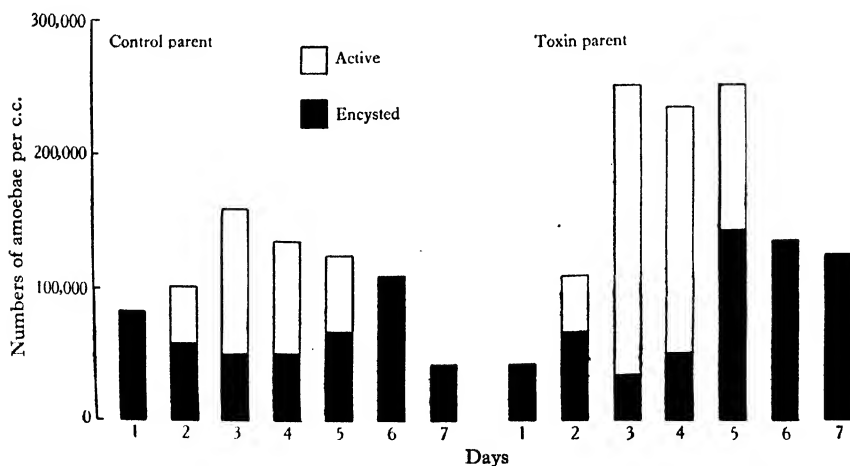


Fig. 4. Cultures prepared after second centrifuging and boiling by inoculating the cysts from the control and toxin cultures into fresh hay infusion.

determining causes of encystation is often encountered, but various considerations make this untenable.

In certain Protozoa it is known that the formation of cysts plays a regular part in the life cycle of the animal. For example, in *Oicomonas termo* when two individuals conjugate the result is the formation of a cyst, from which a single active animal emerges; while in *Colpoda cucullus* and many other ciliates true reproductive cysts occur as part of the life cycle, the encystation of one animal results in the production of four or more others. Another type of cyst also occurs as a regular episode in the life history of *Amoeba diploidea* where, instead of two individuals conjugating, fusion takes place between the two halves of an animal which has itself just divided within the cyst.

Table IV. Average daily number of bacteria and three species of Protozoa for each month in the year 1922. (Protozoa given to the nearest hundred per gramme of soil.)

Month	Bacteria in millions per gm. of soil	Dimastigamoeba		Oicomonas		Heteromita		Cercomonas	
		Total	% Active	Total	% Active	Total	% Active	Total	% Active
January	26	2319	63	879	52	3299	60	8	77
February	24	1514	57	1142	54	2080	64	5	69
March	23	1311	62	1208	51	2218	59	1	68
April	30	2010	66	503	53	3315	66	1	65
May	32	1869	67	961	55	3092	62	1	69
June	37	952	44	1101	45	3243	58	1	70
July	19	169	33	530	50	1079	56	58	69
August	17	121	32	896	63	1253	57	186	69
September	18	344	53	1153	51	1681	49	83	71
October	18	1010	41	733	47	1696	51	71	74
November	41	2111	65	1232	56	3000	57	102	78
December	34	2004	44	902	50	2787	56	38	68

✓ Indirect evidence that cyst formation is a regular occurrence in the life history of free-living Protozoa is afforded by their behaviour in soil, where there are a number of species of amoebae and flagellates continually altering in numbers and playing their part in the soil economy (1). That they are successful members of the population and largely independent of the changing soil conditions is evidenced by the large numbers which are present throughout the year (Tables IV and V). It is impossible to imagine that such an average percentage of cysts, built up as it is from records of daily observations, when the numbers of cysts may fluctuate from 0 to 100 per cent. of the total numbers, can be explained on any assumption but that encystment is a normal stage in the animal's life history, though certain external conditions will undoubtedly help to determine the time at which the cysts are produced. The truth of this can be seen in the *B. mycoides* culture where the cysts were produced at an earlier date than in the control. Further, a consideration of Figs. 1, 2, 3 and 4 suggests that during an amoeba's career it may at any moment be faced with conditions both internal and external which make further repro-

duction impossible and compel it to encyst or die; such occasions are seen in Fig. 1 on the third and fourth days, and in Fig. 4 on the fifth day of the toxin culture.

Encystment cannot be an easy process because if this were the case one would expect the whole of a culture to form cysts, without any appreciable drop in

Table V. *Average daily percentage of cysts of Naegleria gruberi for each month compared with the average daily temperature, moisture and rainfall.*

Month	Cysts	Minimum soil temperature in ° F. 12 in. thermometer	Percentage moisture	Rainfall in inches
January	36	43	23	0·007
February	42	39	22	0·006
March	38	43	19	0·031
April	34	46	19	0·049
May	33	54	17	0·004
June	55	59	14	0·006
July	64	59	21	0·013
August	68	58	21	0·004
September	47	56	20	0·007
October	55	51	22	0·004
November	39	43	22	0·056
December	58	40	23	0·077

numbers; but since death nearly always accompanies encystment it seems more likely that it is a matter of delicate adjustment; and unless the animal's internal condition, either physiological or morphological, coincides with the appropriate condition of the medium the cyst cannot be formed and death inevitably follows.

SUMMARY.

1. Filtrates prepared from young cultures of "YB" bacteria and of *B. prodigiosus* do not depress the rate of growth of *Hartmanella hyalina* appreciably.
2. A filtrate prepared from disintegrated cells of *B. mycoides* inhibits reproduction in *Naegleria gruberi*, and also hastens the onset of cyst formation.

REFERENCES.

- (1) CUTLER, D. WARD, CRUMP, L. M. and SANDON, H. (1922). "A quantitative investigation of the bacterial and protozoan population of the soil, with an account of the protozoan fauna." *Phil. Trans. B*, **211**, 317-50.
- (2) CUTLER, D. WARD and CRUMP, L. M. (1927). "The qualitative and quantitative effects of food on the growth of a soil amoeba (*Hartmanella hyalina*).", *Brit. J. exp. Biol.* **5**, No. 2, pp. 155-65.

FROM THE ANNALS OF APPLIED BIOLOGY, VOL. XXI, No. 3,
pp. 454 -475, AUGUST, 1934.]
[*All rights reserved*]

THE BIOLOGICAL DECOMPOSITION OF PLANT MATERIALS

PART IX. THE AEROBIC DECOMPOSITION OF HEMICELLULOSES

By A. GEOFFREY NORMAN, D.Sc., F.I.C.

(*From the Rothamsted Experimental Station, and the Department of
Agricultural Bacteriology, University of Wisconsin, U.S.A.*)

CONTENTS.

	PAGE
I. Introduction	454
II. Experimental	456
(i) Hemicellulose-decomposing fungi	457
(ii) Hemicellulose-decomposing bacteria	458
Morphological characteristics	460
(a) Mesophilic organisms	460
(b) Thermophilic organisms	464
Biochemical reactions	465
Identification of organisms	467
Fermentation experiments	468
III. Discussion	471
IV. Summary	473
V. Acknowledgments	474
References	474

I. INTRODUCTION.

THE decomposition of hemicelluloses by micro-organisms has, until quite recently, received little attention, even though representatives of this rather indefinite group of polysaccharides are present to a greater or lesser extent in practically all types of plant materials. Not infrequently, next to cellulose, do they form the largest carbohydrate constituent, and accordingly their availability may markedly influence the rate of decomposition of the tissue as a whole. Their importance in industrial fermentations involving natural materials seems often to have been overlooked. Waksman and Diehm(7) have recently reviewed in a detailed manner the literature dealing with the distribution and degradation of the hemicelluloses under natural conditions, and no useful purpose would be served by repeating these citations.

Very few investigations of the decomposition of isolated hemicelluloses in a pure state have been recorded, partly because their preparation in a satisfactory condition presents some difficulties, and partly because accurate methods of estimation and, therefore, of following the decomposition are lacking. Though there are some cases in which there is only one sugar unit involved, more generally two and sometimes three sugars are present. Both hexoses and pentoses are commonly present together with a hexose uronic acid. These form the polyuronides which are the common cell wall encrusting materials in most plant materials and woods. Associated intimately with the cellulose, and isolated with it, is another form of hemicellulose, free or almost free of uronic acid units, and in general of only one sugar unit. These have been termed cellulosans(1). Their rôle is different to that of the encrusting polyuronides, for they are an integral part of the cellulose framework of the plant. In most materials the polyuronides are quantitatively more important than the cellulosans, which are presumably distributed evenly throughout the cellulose and therefore cannot be utilised to any extent unless the cellulose also is attacked. Preparations made by the alkaline extraction of materials, such as straw, contain both polyuronides and cellulosans, and there is as yet no method of effecting a separation. Since the polyuronides are the more soluble, they usually predominate. The linkage in both classes of hemicellulose seems to be of a similar nature and, from a chemical point of view, relatively strong and fairly resistant to mild acid hydrolysis. Hemicelluloses are distinctly more stable towards acids than starch, glycogen or pectin, but much less stable than true cellulose. The structural heterogeneity of individuals of this group raises interesting possibilities of the unequal assimilation of constituent units. That they are readily available to micro-organisms is clear from the work of Rege(4), the earlier work of Waksman (both of which workers only estimated the furfural yielding groups, expressing these rather unfortunately as pentosans), and from that described in earlier papers of this series in which an attempt was made to differentiate between the various furfural-yielding constituents and groups of the hemicelluloses. Not only are they extensively attacked, but their removal is accomplished soon after decomposition has set in, and before the main bulk of the cellulose is lost. In a normal aerobic rot with a mixed natural flora the hemicelluloses seem to provide abundant readily available material for the development of a large and active flora, thus paving the way for the attack on the cellulose, which in materials deficient in hemicelluloses or from which this fraction has been removed, is notably slower. Observa-

tions on mixed flora decompositions and indirect evidence from nitrogen immobilisation figures suggest that fungi develop first and probably are responsible for much of the observed loss of hemicelluloses. Accordingly in the work to be described many fungi were tested as to their ability to utilise a natural hemicellulose preparation, and a search made for aerobic bacteria which would also do so.

Whilst this work was in progress Waksman and Diehm(8,9) published the results of a study on somewhat similar lines. They employed as substrates preparations of so-called "pentosan" from Dulse (a seaweed), mannan, galactan, and xylan, only the last of which could reasonably be regarded as pure. It has yet to be shown that the pentosan from Dulse, or the galactan from Irish Moss, are similar in composition to the hemicelluloses of the higher plants, and since the total hemicellulose in the galactan amounted to only a little over 50 per cent., and in the mannan and pentosan to less than 75 per cent., there is no certainty that any hemicellulose was really utilised when only a partial fermentation occurred. A number of organisms isolated from an enrichment culture in the "pentosan" were unable to utilise xylan. Similarly, since the preparations with one exception were not nitrogen free, the figures for the amount of carbohydrate decomposed per unit of nitrogen assimilated are not absolute values, for all, or a part, of the organic nitrogen of the preparation may also have been utilised. In spite of these objections to the quantitative aspects of their survey, these workers have shown that there is in the soil an abundant flora of all types capable of utilising hemicelluloses to some extent.

II. EXPERIMENTAL.

The source of the crude hemicellulose preparation used in this work was oat straw, from which it was prepared by the usual means, namely extraction with warm 4 per cent. NaOH after pretreatment with alcoholic soda to remove a portion of the lignin. The extract was acidified with acetic acid after filtration through glass-wool and sufficient alcohol added for complete precipitation. The precipitate was purified by solution in alkali and reprecipitation. A portion was also fractionated into preparations A and B, the former by the addition of an excess of glacial acetic acid to the crude solution, and the latter by the addition of alcohol or acetone to the acetic acid filtrate. All preparations were low in ash and nitrogen free. The crude preparation used in most of the work to be described gave 2.04 per cent. CO_2 from the uronic acid present, and 34.0 per cent. furfural from the pentose and uronic groups. This corre-

sponds approximately with 8.2 per cent. uronic acid and 50.8 per cent. pentose, expressed as xylan, the remaining 41 per cent. presumably being hexosan.

(i) *Hemicellulose-decomposing fungi.*

That some fungi are capable of utilising the hemicelluloses of natural materials has been known for some time, and shown directly by the work of Waksman (6) and Norman (3) in pure culture. They have further been suspected of playing a large part in the removal of this group in rots brought about by a mixed natural flora of bacteria and fungi together. There is under ordinary conditions a rapid removal of the hemicelluloses in the early stages of fermentation, and coincident with this there is observed to be a very considerable development of fungal tissue. In general, fungi seem to be less specialised than most bacteria in their nutritional requirements. Materials such as straw rotted by a single fungus give on analysis figures very similar to those obtained on partial fermentation with a mixed flora, and all groups of plant constituents are attacked. Therefore it seemed likely that many fungi can utilise hemicelluloses, and that the isolation and identification of well-known forms from enrichment cultures would not be of much value. Accordingly it was decided to make a qualitative test of the utilisation of hemicelluloses by a large group of common fungi. Of all groups in nature the *Aspergilli* and *Penicillia* seem to be the commonest and most ubiquitous. Nearly seventy organisms were tested, many of them being of one or the other of these groups.¹ They were transferred from slopes on glucose-peptone to Petri plates with medium of the following composition: agar 15 gm., hemicellulose 10 gm., peptone 5 gm., $(\text{NH}_4)_2\text{HPO}_4$ 1 gm., KCl 0.5 gm., MgSO_4 0.5 gm., Fe salt trace, per litre, $\text{pH} = 7.0$. The hemicellulose goes into solution on autoclaving and the medium on cooling is opalescent and creamy brown in colour. No hydrolysis takes place under this treatment and the medium is non-reducing. The inoculation was a small one on the centre of the plate and done in quadruplicate. At the same time four other plates were set up with a medium similar in composition, except that glucose was substituted for the hemicellulose, and two plates with a medium containing the other constituents but no carbohydrate. This was to distinguish between growth at the expense of carbohydrate and growth on the peptone, the concentration of which was rather high. At the end of two days and a week the growth was compared, the colony diameter measured and general appearance as-

¹ I am indebted to Dr H. C. Greene for these cultures.

sessed. In some cases there was a marked ring or cleared zone round the colony on the hemicellulose medium indicating the production of an exo-enzyme. On the medium containing peptone and inorganic salts alone growth was in all cases meagre, scanty, or nil. Table I gives the comparative development on glucose and hemicellulose medium at the end of 2 and 7 days.

The most striking feature of the results obtained was the fact that every organism which was tested grew reasonably well on the hemicellulose medium, the majority at least as well as on glucose, and quite a number better than on glucose. Inasmuch as the cultures tested were selected entirely at random this was somewhat surprising and indicates that the ability to utilise hemicelluloses of this type is widespread amongst fungi. The cleared zone round the colony was a prominent feature on many plates. In general this appeared quite soon after inoculation and sometimes became less obvious as the colony enlarged. Those cases marked † in Table I still exhibited a wide cleared area at the end of 7 days, when in most cases the colony had reached almost its maximum growth. This property of the production of an exo-enzyme might possibly be of value in distinguishing between certain closely allied *Penicillia* or *Aspergilli*.

(ii) *Hemicellulose-decomposing bacteria.*

The method of approach in the case of bacteria was somewhat different and, instead of testing known organisms for their ability to decompose hemicelluloses, isolations were made from various natural sources.

The following media were employed:

Medium HA, per litre:—20 gm. purified hemicellulose (oat straw), 5 gm. peptone, 1 gm. KH_2PO_4 , 0.5 gm. KCl , 0.5 gm. MgSO_4 , trace FeSO_4 , 15 gm. agar, $\text{pH} = 7.6$.

Medium HAL. The above without the addition of agar.

Medium IIB, per litre:—10 gm. purified hemicellulose, 1.0 gm. NH_4NO_3 , 1.0 gm. K_2HPO_4 , 0.5 gm. MgSO_4 , 0.5 gm. NaCl , 0.5 gm. peptone, trace FeSO_4 , 15 gm. agar, $\text{pH} = 7.0$.

Medium HBL. The above without the addition of agar.

From soil enrichment cultures, decomposing straw, and manure a considerable number of aerobic organisms were isolated. Inasmuch as non-hemicellulose decomposing organisms will develop on the peptone in medium HA, the following criteria are necessary and were applied. To be regarded as a hemicellulose decomposer an organism must either

Table I.
Growth of fungi on hemicellulose medium.

Organism	2 days	7 days
Aspergilli		
<i>Aspergillus</i> sp. 4235 X 4 Thom	$H = G$	$H > G^*$
<i>A. fumigatus</i>	$G > H$	$H > G^*$
<i>A. ochraceus</i> 4700 a	$G > H^*$	$H = G$
<i>A. oryzae</i> No. 965	$G > H$	$H > G^\dagger$
<i>A. parasiticus</i> 3509	$H = G^*$	$G > H$
<i>A. schiennanni</i> 3534 C	$G > H^*$	$G > H$
<i>A. spadic</i> 4707 757	$H = G^*$	$H = G$
<i>A. sydowi</i> 5128 5114 Thom	$H = G^*$	$H = G$
<i>A. tamaris</i> 4735	$H = G$	$H = G$
<i>A. terricola</i> var. <i>americana</i> 4838	$H = G$	$G > H$
<i>A. ustus</i> 3556	$H = G$	$G > H^*$
<i>A. wentii</i> 44173	$H = G$	$H = G$
<i>Aspergillus</i> sp. Taka. N. 292 Thom	$H = G^*$	$G > H^*$
<i>Aspergillus</i> sp. 1 (? <i>niger</i>)	$H = G^*$	$H > G$
<i>Cephalothecium</i> sp.	$H = G^*$	$G > H^*$
<i>Clonostachys araucaria</i> 5128 5113 Thom	$H = G^*$	$H = G$
<i>Coprinus radians</i>	$G > H$	$G > H^*$
<i>Cunninghamella</i> sp.	$G > H$	$H = G$
<i>Fomes annosus</i>	$H = G$	$H = G$
Gliocladia		
<i>G. atrum</i> 4894 18 Thom	$G > H$	$H = G$
<i>G. deliquescentis</i> 5128 5101, 1 Thom	$G > H$	$H = G$
<i>G. fimbriatum</i> 5128 5101, 3 Thom	$G > H$	$H = G$
<i>G. roscum</i> 5128 5101, 2 Thom	$H = G$	$H > G^*$
<i>G. vernoosenii</i> 5090 4 a Thom	$H = G$	$G > H^*$
<i>Leucites lepidus</i>	—	$H = G$
<i>Monascus</i> sp.	$H = G$	$H = G$
<i>Monilia sitophila</i>	$G > H$	$H = G$
<i>Mucor</i> sp.	$G > H$	$G > H$
<i>Paeclomyces varioti</i> (syn. <i>Penicillium discaricatum</i>)	$G > H$	$H = G$
Penicillia		
<i>Penicillium anisopliae</i> 1367 Thom	$H = G^*$	$H = G$
(<i>Metarrhizium</i>)		
<i>P. atramentosum</i> 4733 3 Thom	$G =$	$G > H$
<i>P. aurantio-brunneum</i> 4733 5 Thom	$G > H$	$H = G^\dagger$
<i>P. avellaneum</i> 4401 Thom	$H = G$	$H = G$
<i>P. cascicolum</i> Hastings 1, 17, A	$H = G$	$H = G$
<i>P. chloro-leucon</i> 4733 30 Thom	$H = G$	$H = G^\dagger$
<i>P. chrysogenum</i> 4733 33 Thom	$H = G$	$H = G$
<i>P. chrysoscezi</i> 5101 7 Thom	— —	$H = G$
<i>P. citreo-viride</i> 4733 38 Thom	$H = G$	$G > H$
<i>P. citrinum</i> 4482 Thom	$H = G^*$	$H = G^\dagger$
<i>P. commune</i> 23 Thom	$H = G$	$G > H$
<i>P. cyaneo-fulvum</i> 4733 47 Thom	$H = G$	$H = G^\dagger$
<i>P. cyanum</i> 4640 422 Thom	$H = G^*$	$H = G^*$
<i>P. digitatum</i>	$G > H$	$G > H^\dagger$
<i>P. elegans</i> 5129 B Thom	$H = G$	$H > G$
<i>P. expansum</i> 1, H. Greene	— —	$H = G$
<i>P. granulatum</i> 4641 Thom	$G > H$	$G > H$
<i>P. granulatum</i> 1, H. Greene	$H = G$	$H = G$
<i>P. Hayami</i> 5010 8 Thom	$H = G$	$H > G^*$
<i>P. islandicum</i> 4785 7 Thom	$H = G^*$	$G > H^*$
<i>P. janthinellum</i> 4141 Thom	$H = G$	$\dagger G > H^\dagger$
<i>P. Jenseni</i> 5010 10 Thom	$H = G^*$	$G > H^\dagger$
<i>P. Johannioli</i> 5010 11 Thom	$G =$	$H = G^*$
<i>P. lilacinum</i> 4855 Thom	$H = G$	$H = G$
<i>P. luteum</i> 1124	$G > H$	$G > H^*$

Table I (cont.).

	Organism			2 days	7 days
<i>P. matris-mecae</i>	5010	14	Thom	$G > H$	$H > G$
<i>P. oxalicum</i>	Soil		Thom	$G > H$	$H > G^*$
<i>P. purpureogenum</i>	1128			$G > H$	$H = G^\dagger$
<i>P. Racidorskii</i>	5010	19	Thom	$G =$	$H = G$
<i>P. roqueforti</i>	4733	102	Thom	$G > H$	$H = G$
<i>P. sanguineum</i>	4917	7	Thom	$G > H$	$H > G^*$
<i>P. solitum</i>	2546		Thom	$H = G$	$H = G$
<i>P. spinulosum</i>	45		Thom	$G > H$	$G > H$
<i>P. terrestre</i>	5034	8	Thom	$H = G$	$G > H$
<i>P. verrucosum</i>	4733	125	Thom	$H = G$	$H = G$
<i>Rhizopus nigricans</i>				$G > H$	$G > H$
<i>Sclerotium rolfsii</i>				$H > G$	$H > G^\dagger$
<i>Syncephalastrum</i> sp.				$H = G$	$H > G$

KEY: $H > G$, organism developed better on hemicellulose than on glucose.

$H = G$, organism developed as well on hemicellulose as on glucose.

$G > H$, organism developed better on glucose than on hemicellulose.

* Cleared zone round colony on hemicellulose medium.

† Very marked clearing.

‡ Sectoring of colony.

grow on medium HB, produce a clearing of the semiopaqueness of media HBL and HAL, or show a cleared zone round the colony on medium HA. Not every organism which clears medium HAL will produce such a zone on the agar medium HA. In point of fact every organism described grew to a greater or lesser extent on the liquid medium HBL, though some developed more actively when supplied with nitrogen in an organic form. Isolations were made at 30 and 65° C. The following seventeen mesophilic organisms, five of which are closely related species, and three thermophilic organisms, were isolated and characterised.

Morphological characteristics.

(a) *Mesophilic organisms.*

No. 1.

Source	Soil.
Medium	Clearing HAL.
Morphology	Rods 2.0-2.5 × 0.5-0.7, occurring often in chains and in bundles. Many spores at 42 hours. No swelling. Spore size 2.0-2.5 × 1.0-1.2, thickened at end.
Gram stain	Strongly positive.
Gelatin stab	Liquefaction.
Agar plates	Circular, smooth, flat, edge lobate, very coarsely granular.
Agar plants	Spreading, flat, dull, smooth, translucent, no colour, consistency butyrous, medium unchanged. Visible growth 4 hours.
Agar shake tubes	Aerobic.
Broth	Surface growth and pellicle 12 hours, heavy sediment 2 days.
Litmus milk	Reduction of litmus 5 days, peptonisation by 12 days, no coagulation.
Potato	White, spreading.
Catalase	Positive, very strong.

No. 2.

Source	Soil.
Medium	Clearing HAL.
Morphology	Rods $1.6-2.0 \times 0.5-0.7$, arranged in bundles. Spores few at 60 hours. Spore size 1.0×2.5 . No swelling.
Gram stain	Negative.
Gelatin stab	Liquefaction.
Agar plates	Circular, smooth, flat, edge lobate, coarsely granular.
Agar slants	Spreading, flat, dull, smooth, translucent, no colour, consistency butyrous, medium unchanged.
Agar shake tubes	Aerobic.
Broth	Surface growth and light pellicle, 12 hours. some sediment 2 days.
Litmus milk	Slight reduction of litmus by 60 hours, peptonisation by 12 days, no coagulation.
Potato	Thick, white, gummy.
Catalase	Positive, strong.

No. 3.

Source	Soil.
Medium	Clearing HAL.
Morphology	Rods $1.6-2.0 \times 0.8-1.0$, occurring in bundles. Spores at 60 hours. No swelling. Spore size 1.0×2.0 , end thickened.
Gram stain	Strongly positive.
Gelatin stab	Liquefaction.
Agar plates	Circular, smooth, flat, edge undulate, finely granular.
Agar plants	Spreading, flat, dull, smooth, translucent, no colour, consistency butyrous, medium unchanged.
Agar shake	Aerobic.
Broth	Surface growth, some flocculum 12 hours, pellicle, 2 days.
Litmus milk	Coagulation 36 hours, complete peptonisation 9 days.
Potato	Thick, white, gummy.
Catalase	Positive, very strong.

Nos. 4, 6, 12, 14, 15.

Source	4 and 6 soil; 12, 14, and 15 rotting straw.
Medium	HBL.
Morphology	Short rods 1.5×1.0 and smaller. No spores. Rods become smaller and coccoid with time.
Gram stain	Negative.
Gelatin stab	No liquefaction.
Agar plates	Circular, smooth, raised (concave). Edge entire to slightly undulate becoming lobed, amorphous, star forms in centre of colony.
Agar slants	Growth abundant, echinulate, raised, glistening opalescent, no colour, consistency viscid, medium unchanged.
Agar shake	Aerobic, facultative, gas evolution.
Broth	Turbid, gas evolution and pellicle at 12 hours, flocculant ring 2 days.
Litmus milk	Slight acidity 12 hours, acid and gas 36 hours, reduction of litmus and coagulation 60 hours, gas production but no peptonisation.
Potato	4 and 14, spreading. 6, thick whitish, heavy. 12, restricted, gummy. 15, whitish, wrinkled.
Catalase	4, positive, weak. 12, positive, very strong. 6, 14, 15, positive, strong.

No. 5.

Source	Soil.
Medium	Clearing HAL.
Morphology	Rods $1.5-2.5 \times 0.5-0.8$ in definite chains. Spore formation without change of shape at 24 hours. Spore size $1.5-2.0 \times 1.0-1.2$.
Gram stain	Negative.
Gelatin stab	Liquefaction.

462 *The Biological Decomposition of Plant Materials*

Agar plates	Circular, smooth, raised margin, edge vague, granular.
Agar slants	Spreading, flat, dull, smooth, translucent, no colour, consistency butyrous, medium unchanged.
Agar shake	Aerobic.
Broth	Turbid with pellicle, 12 hours, some sediment, 2 days.
Litmus milk	Reduction of litmus and coagulation 36 hours, complete peptonisation, 9 days.
Potato	Yellowish, moist.
Catalase	Positive, weak.

No. 7.

Source	Soil.
Medium	Clearing HAL.
Morphology	Rods $1.5-2.0 \times 0.5-0.8$ in bundles. Many show granulations. Spores at 42 hours. Spore size $2.0-2.5 \times 1.0$. Thickened at ends.
Gram stain	Positive.
Gelatin stab	Liquefaction.
Agar plates	Spreading, smooth, flat, deep lobes, granular.
Agar slants	Spreading, flat, dull, smooth, translucent, no colour, consistency butyrous, medium unchanged.
Agar shake	Aerobic.
Broth	Pellicle 12 hours, some sediment 2 days.
Litmus milk	Reduction of litmus, coagulation and slow peptonisation.
Potato	White, heavy.
Catalase	Positive, strong.

No. 8.

Source	Manure.
Medium	Clear zone on HA.
Morphology	Rods $2.5 \times 0.5-0.7$, packed in bundles. Occasional pleomorphic forms up to 15 long. Spores rod-shaped at 84 hours. Spore size $1.0-2.0 \times 0.8$.
Gram stain	Negative, or slightly positive.
Gelatin stab	Slight liquefaction.
Agar plates	Spreading, slightly rough, edge irregular and thickened, faintly granular.
Agar slants	Growth moderate, echinulate, flat, dullish, contoured, translucent, no colour, consistency soft, medium unchanged.
Agar shake	Aerobic.
Broth	Turbidity at surface, 12 hours, general turbidity 2 days.
Litmus milk	No change to 6 days, some coagulation, and slight peptonisation 12 days.
Potato	Whitish, wrinkled.
Catalase	Positive, strong.

No. 9.

Source	Manure.
Medium	Clear zone on HB.
Morphology	Rods $2.0-2.5 \times 0.5-0.8$ showing clearly defined granulations. Spores at 60 hours, thickened at ends. Spore size $1.8-2.0 \times 1.0-1.2$.
Gram stain	Negative.
Gelatin stab	Liquefaction.
Agar plates	Spreading, smooth, flat, edge deeply lobed—irregular, coarsely granular.
Agar slants	Spreading, flat, dull, smooth, translucent, no colour, consistency dry, medium unchanged.
Agar shake	Aerobic.
Broth	Surface growth and pellicle, 12 hours.
Litmus milk	Coagulation by 36 hours, strong peptonisation, complete by 9 days.
Potato	White, very wrinkled.
Catalase	Positive, weak.

No. 10.

Source	Manure.
Medium	Clearing HAL.
Morphology	Rods $2.0-2.5 \times 0.5-0.8$, and occasionally longer. Spores at 84 hours, thick walled. Spore size $1.6-1.8 \times 0.8-1.0$.
Gram stain	Positive?

Gelatin stab	Liquefaction.
Agar plates	Spreading, smooth, flat, edge deeply lobed, very coarsely granular.
Agar slants	Spreading and arborescent, flat, dull, smooth, translucent, no colour, consistency butyrous, medium unchanged.
Agar shake	Aerobic.
Broth	Surface growth and pellicle with some sediment, 12 hours, sediment 2 days.
Litmus milk	Reduction of litmus, coagulation 5 days, peptonisation steady but slow.
Potato	White, very wrinkled.
Catalase	Positive, very strong.

No. 11.

Source	Manure.
Medium	Clearing HAL.
Morphology	Rods 2.0-2.5 × 0.5-0.8, grouped in bundles. Spores at 42 hours without swelling. Spore size 1.6 × 1.0.
Gram stain	Negative.
Gelatin stab	Liquefaction.
Agar plates	Spreading, smooth, flat, edge deeply lobed and arborescent, very coarsely granular.
Agar slants	Spreading, flat, dull, thin, translucent, no colour, consistency butyrous, medium unchanged.
Agar shake	Aerobic.
Broth	Surface growth and pellicle 12 hours; sediment 2 days.
Litmus milk	Reduction of litmus, and slow peptonisation from 6 days.
Potato	Slight growth.
Catalase	Positive, weak.

No. 13.

Source	Manure.
Medium	Clearing HBL.
Morphology	Long rods 3.0-4.5 × 0.5-1.0. Some pleomorphic forms up to 8. Spores at 42 hours, without swelling. Spore size 1.6-1.8 × 1.0.
Gram stain	Positive.
Gelatin stab	Liquefaction.
Agar plates	Irregular, smooth, flat, edge lobate, coarsely granular, or filamentous.
Agar slants	Spreading, flat, dull, thin, translucent, no colour, consistency butyrous to dry, medium unchanged.
Agar shake	Aerobic.
Broth	Faint turbidity 12 hours, pellicle, 2 days.
Litmus milk	Reduction of litmus and coagulation in 60 hours, slow peptonisation.
Potato	Whitish, wrinkled.
Catalase	Positive, weak.

No. 16.

Source	Manure.
Medium	Clearing HAL.
Morphology	Rods 2.0-2.5 × 0.5-0.8. Marked granulations. Spores at 42 hours, central without swelling. Spore size 1.5-1.8 × 0.6-0.8.
Gram stain	Negative. Few rods positive.
Gelatin stab	Liquefaction.
Agar plates	Circular, smooth, flat, edge lobate.
Agar slants	Spreading and arborescent, flat, glistening, smooth, translucent, no colour, consistency butyrous, medium unchanged.
Agar shake	Aerobic.
Broth	Surface growth and pellicle 12 hours, sediment 2 days.
Litmus milk	Reduction of litmus and coagulation in 60 hours, steady peptonisation.
Potato	White, very heavy growth.
Catalase	Positive, strong.

No. 17.

Source	Manure.
Medium	Clearing HAL.
Morphology	Rods 3.0-3.5 × 1.0. Granulation. No spores.

464 *The Biological Decomposition of Plant Materials*

Gram stain	Negative.
Gelatin stab	Liquefaction.
Agar plate	Circular, smooth, flat, edge undulate, strongly granular, filamentous.
Agar slants	Growth moderate, separate groups, flat, glistening, smooth, translucent, no colour, consistency viscid, medium unchanged.
Agar shake	Aerobic.
Broth	Some turbidity 12 hours, turbidity 2 days, no pellicle or sediment.
Litmus milk	Coagulation in 36 hours, slight acid 60 hours, reduction of litmus and strong peptonisation.
Potato	White, very heavy growth.
Catalase	Positive, very strong.

(b) *Thermophilic organisms.*

X.

Source	Soil.
Medium	Clearing HA and HB.
Morphology	Long rods, 5.0-9.0 \times 1.0, often with terminal spores swelling to club shape or even plectridium form. Size variable on liquid media, more constant on agar. Median band visible occasionally. Spore size 1.6 \times 1.0 approximately.
Gram stain	Negative.
Gelatin	(Frazier method—tannic acid.) Liquefaction.
Agar plate	Spreading, thin, gummy.
Agar shake	Aerobic.
Broth	Turbid, no pellicle.
Litmus milk	Coagulation and some peptonisation.
Potato	Gummy growth.
Catalase	Negative, or very faint.
Starch agar	Good growth, marked clear zone round colonies.

Y.

Source	Manure.
Medium	Clearing HA.
Morphology	Long rods, 16.0-12.0 \times 1.0. Variable in length, no swelling or visible spores, marked granulation, cells sometimes convoluted. Very pleomorphic in liquid culture.
Gram stain	Negative.
Gelatin	(Frazier method—tannic acid.) Liquefaction.
Agar plate	Spreading, thin, gummy.
Agar shake	Aerobic.
Broth	Turbid, no pellicle.
Litmus milk	Reduction of litmus. Coagulation and peptonisation 3 days, heavy peptonisation by 9 days.
Potato	Gummy growth.
Catalase	Negative.
Starch growth	Good growth, marked clear zone round colonies.

Z.

Source	Manure.
Medium	Clearing HA. and HB., preferring the latter.
Morphology	Very long rods resembling actinomycetes in liquid culture. Some shorter rods 5.0 \times 1.0 and less. More constant on agar. No visible spores. Some granulation.
Gram stain	Positive.
Gelatin	(Frazier method.) No liquefaction.
Agar plate	Spreading, thin, gummy.
Agar shake	Aerobic.
Broth	Turbid, no pellicle.
Litmus milk	Coagulation and marked peptonisation 7 days.
Potato	Gummy growth.
Catalase	Negative.
Starch agar	Good growth, marked clear zone round colonies.

*Biochemical reactions.**(a) Utilisation of nitrogen.*

Medium: glucose 10 gm., K_2HPO_4 0.5 gm., NaCl 0.5 gm., $MgSO_4$ 0.2 gm. per litre, $pH=7.0$.

1 gm. each per litre of KNO_3 , KNO_2 , ammonium phosphate, urea, and peptone added, the nitrite being sterilised separately and added to the tubes after sterilisation.

Brom-chlor-phenol-blue (range pH 3.0-4.8) was also added to the peptone and ammonium phosphate series in the case of the mesophilic organisms.

Organism	Nitrate*		Nitrite	Ammonium phosphate		Urea	Peptone	
	Growth	Reduction to nitrite		Growth	pH 1 day		Growth	pH 1 day
1	-	-	-	+	No change	+	+	No change
2	+	+	-	+	"	+	+	"
3	+	Trace	-	+	"	+	+	"
4, 6, 12, 14 and 15	+	++	-	+	4.0	+	+	3.5-4.0
5	+	+	-	+	+ gas No change	+	+	+ gas No change
7	-	-	-	+	"	-	+	"
8	-	-	-	-	"	-	+	"
9	+	-	-	+	"	+	+	"
10	+	Trace	-	+	"	+	+	"
11	+	Trace	-	+	"	+	+	"
13	+	-	-	+	"	+	+	"
16	-	-	-	+	"	+	+	"
17	-	-	-	-	"	+	+	"
X	+	+	-	-	-	-	+	-
Y	-	-	-	-	-	-	+	-
Z	+	+	-	+	-	-	+	-

* When tested for nitrate reduction on the standard medium used for that test (glucose 2.0 gm., beef extract 2.5 gm., peptone 1.0 gm., KH_2PO_4 1.0 gm., KNO_3 1.0 gm. per litre) all organisms developed well and the pink colour indicative of nitrite, on addition of α -naphthylamine in acetic acid and sulphanilic acid, was given on plates by all except 7 and 8 and in broth by all except 7, 8, X, Y and Z. Some organisms which are unable to utilise and reduce nitrate when supplied with that alone, can seemingly do so when supplied as well with an organic source of nitrogen.

(b) Indole production.

No organism gave a positive test for indole when growing on a medium consisting of 1 per cent. peptone and 0.5 per cent. NaCl, either at 1 day or after 14 days' incubation.

(c) H_2S production (Kligler's lead acetate method).

Negative in all cases at 1 day and 1 week.

(d) Reduction of methylene blue.

At 1 day, marked reduction by 3 and 9, slight by 2, and at 4 days complete reduction by 2, 3 and 9.

466 *The Biological Decomposition of Plant Materials*

(e) *Utilisation of carbohydrates.*

(i) *Gas production from glucose-peptone broth (1 per cent. glucose).* Considerable gas was given by the facultative group Nos. 4, 6, 12, 14 and 15, and only small quantities by any other organism.

(ii) *Acid production from glucose peptone broth (1 per cent. glucose).* Five c.c. of the broth used for the gas production test above were titrated with *N/10* alkali after fermentation. The facultative group, 4, 6, 12, 14 and 15, gave a titration of about 0.7–0.8 c.c. *N/10* alkali, while no other organism produced more than a trace of acid.

(iii) *Availability of sugars and polysaccharides.* The organisms were inoculated into a set of tubes in duplicate, containing a liquid medium of 1 per cent. carbohydrate together with 0.1 per cent. peptone and the usual mineral salts. The growth was observed, and the acid produced titrated with *N/10* alkali at the end of 2 weeks. Every organism grew on every carbohydrate tested, but in few cases was there more than a trace of acid produced. In many cases there was either a sediment, or pellicle in addition to turbidity of the medium. No useful purpose would be served by giving the titrations in detail. The facultative group Nos. 4, 6, 12, 14 and 16 gave small quantities of acid from a number of sugars (in quantity up to the equivalent of 1.0 c.c. *N/10* alkali per 5 c.c. culture solution). Most of the organisms showed some acidity on starch and salicin, and a few on glucose and sucrose. The carbohydrates tested were: arabinose, fructose, galactose, glucose, lactose, maltose, mannitol, melizitose, α -methylglucoside, pectin, raffinose, rhamnose, salicin, starch, sucrose, and xylose.

In view of the ability of these organisms to utilise to some extent such a wide range of carbohydrates, it was hoped to effect some differentiation by presenting them with certain mono- and disaccharide acids of known linkage, and some less available polysaccharides. The following acids were employed in the form of the calcium salt:

- (i) Gluconic acid,
- (ii) Saccharic acid,
- (iii) Maltobionic acid (4 α -glucosido-gluconic acid),
- (iv) Lactobionic acid (4 β -galactosido-gluconic acid),
- (v) Tetragalacturonic acid¹ (+ CaCO₃ when sterile),

and the following other substances; gum arabic, mesquite gum, glycerol, and starch. The medium in all cases but the last was a broth containing 1 per cent. carbohydrate, 0.1 per cent. peptone, 0.05 per cent. ammonium

¹ I am indebted to Prof. K. P. Link for this acid.

phosphate. The starch was used in an agar medium of similar composition on plates, the better to observe any zoning round the colonies.

The growth on these carbohydrates is given in Table II. It will be seen that glycerol, starch, and the two gums, were fermented by all the organisms. Similarly tetragalaturonic acid was utilised by all, though not to any great extent. This was not unexpected, inasmuch as pectin, of which molecule tetragalaturonic acid forms about 80 per cent., was previously shown to be available.

Gluconic acid, the normal monocarboxylic acid from glucose, was fermented by all organisms, but saccharic acid which has a carboxyl group at each end of the hexose unit was unavailable to all but organisms 8 and 17. These two organisms, however, along with some others, could not utilise maltobionic acid. Lactobionic acid, for no obvious reason, appeared to be more available than maltobionic acid.

Table II.

Utilisation of carbohydrates by mesophilic organisms.

Organism	Gluconic acid		Saccharic acid		Maltobionic acid		Lactobionic acid		Tetra- galaturonic acid		Gum arabic	Mes- quite gum	Glycerol		Starch agar
	1 day	7 days	2 days	11 days	2 days	11 days	1 day	5 days	4 days	10 days	3 days	2 days	1 day	11 days	1 day
1	—	+s	—	—	—	—	+	+p	?	+	+	+	+	+	+ + *
2	—	+ps	—	—	—	—	—	+p	+	+	+	+	+	+	+ + +
3	—	+ps	—	—	—	+	—	+p	+	+	+	+	+	+	+ + +
4, 6, 12, 14, 15	+	+ps	—	—	—	+	+	+	+	+	+	+	+	+s	+
5	—	+ps	—	—	+	+	+	+	+	+	+	+	+	+ps	+ + +
7	—	+ps	—	—	—	+	+	+	+	+	+	+	+	+	+ + *
8	—	+s	+	+	—	—	—	+	+	+	+	+	+	+	+ *
9	—	+p	—	—	—	—	—	—	+	+	+	+	+	+	+ +
10	—	+ps	—	—	—	—	+	+	+	+	+	+	+	+	+ + +
11	—	+p	—	—	—	—	+	+p	+	+	+	+	+	+	+ *
13	—	+p	—	—	—	+	—	—	+	+	+	+	+	+	+ *
16	—	+p	—	—	—	—	+	+p	+	+	+	+	+	+	+ + +
17	—	+p	+	+	—	—	+	+	+	+	+	+	+	+s	+ +

+ = growth; + + = heavy growth; p = pellicle; s = sediment; * = clear zone round colony.

† 4 and 6 fair growth, no adjacent clearing; 14 and 15 fair growth, some clearing; 12 fair growth, good clearing.

Identification of organisms.

The main purpose of this work was to isolate certain organisms capable of utilising hemicelluloses, to determine their activity and general fermentation characteristics, but not necessarily to assign to them a permanent classification. In any case, from the evidence obtained, this would be no easy matter, inasmuch as certain of those isolated do not appear to have been previously described, or else fall in a class in which differentiation is difficult. One group, composed of organisms 4, 6, 12,

14 and 15, stands out from the remainder. These were small rods, Gram-negative, giving no pigment, and forming no spores. They agree closely with the published description of *Achromobacter ubiquitum* (Jordan) originally described as *Bacillus ubiquitus* (2). Several organisms with very similar characteristics have been named separately in this group. The five strains isolated were not identical but showed small morphological and cultural differences notably in growth on potato, and on starch agar plates, and in the strength of the catalase test. Organism 17 possibly falls in the *Achromobacter* group also, though rather above the average size. It does not, however, produce gas from carbohydrates as do those five strains mentioned above.

The remaining organisms were aerobic spore-formers, so common in soil. Not all were Gram-positive, however, and it is possible that the Gram-negative forms represent new species. The cellulose-decomposing organisms of this group, such as *B. amylolyticus* and *B. albus* are Gram-negative and both ferment a rather wide range of carbohydrates producing acid but no gas, as do those isolated in this work. This class of organism deserves further attention.

The thermophilic organisms were not so completely characterised, but in any case identification of members of this group is a difficult and unsatisfactory matter. They exhibited marked pleomorphic characters, so that their purity was not a matter of absolute certainty. Plectridial forms were sometimes found in culture X, while Z had some of the characters of an actinomycete.

Fermentation experiments.

The organisms described were frequently transferred on to media containing hemicellulose lest there should be any loss of activity. While the mixed enrichment cultures from which these were isolated produced a rapid loss of hemicellulose from liquid media, the same could not be said of any of the organisms in pure culture. On media such as HAL or HBL, the faster organisms showed some signs of clearing of the medium at the end of two days. The complete clearing of the opalescent solution was frequently not accomplished in a month, if at all. Various fermentations were carried out with the organisms which seemed to be the more active and rapid. These were notably 3, 7, 8, 13, 16 and 17. The rate of fermentation of a purified sample of hemicellulose A from oat straw by these organisms was determined. To a suspension in water was added per litre: peptone 1 gm., ammonium phosphate 0.5 gm., potassium phosphate 0.5 gm. and a trace of iron. On autoclaving, an

opalescent solution was obtained. About 400 c.c. were fermented at 30° C. in flasks with a side tube which permitted sampling from time to time. At the end of 8, 24 and 40 days about 80 c.c. were withdrawn. Two samples of 10 c.c. were centrifuged to remove sediment, and the residual hemicellulose in each precipitated by the addition of three volumes of alcohol to which a little acetic acid had been added. The precipitate was filtered off, thoroughly washed, and weighed after drying in a vacuum oven at 60° C. and then briefly at 100° C. The assumption that the alcohol precipitate consisted of undecomposed or only slightly degraded hemicellulose is justifiable since the furfural yield of the fermenting solution (which was determined on two 20 c.c. portions) and of the precipitate fell approximately proportionately. In Table III is given the decomposition effected by the organisms selected.

Table III.

Mg. alcohol precipitable hemicellulose in 10 c.c. fermentation liquid.

Initial concentration = 81 mg.

Days	No. 3	No. 7	No. 8	No. 11	No. 16	No. 17
8	72	71	80	74	76	77
24	70	68	76	74	75	74
40	64	66	72	73	73	73

In no case was more than 20 per cent. of the hemicellulose fermented. A mixed culture from soil, on the other hand, in 40 days, left only 8 mg. in a solution initially of the same concentration, a removal of about 90 per cent.

It was mentioned previously that although a wide range of carbohydrates was utilised by these organisms very little acid was produced, and only small quantities of gas, save in the case of those of the *Achromobacter* group. Inasmuch as arabinose and galactose are frequently found in plant hemicellulose, the fermentation of both these sugars by the organisms given above was tested. The residual sugar was determined by the Shaffer-Hartman method, the initial concentration in each case being 1 per cent.

Table IV.

Percentage residual sugar after 2 weeks' incubation at 30° C.

	No. 3	No. 7	No. 8	No. 11	No. 16	No. 17
Arabinose	0.91	0.97	0.96	0.97	1.0	1.0
Galactose	0.91	1.0	0.97	0.96	0.94	0.97

This experiment demonstrated again the apparently feeble nature of these organisms in pure culture. On pectin, however, a rather greater

470 *The Biological Decomposition of Plant Materials*

activity was observed. This was somewhat unexpected, since the units commonly occurring in the hemicelluloses are similar to those in pectin. The linkages of the latter, however, must be of an entirely different nature, inasmuch as they are extremely sensitive to alkaline degradation, while the hemicelluloses are comparatively resistant to alkali. Hydrolysis studies have shown that a solution of pectin, degraded so that no calcium pectate is obtained by the usual method of estimation, may even yet contain polyuronide molecules of considerable size and complexity. Therefore, if in a fermentation trial all pectin is removed, it must not be assumed that all carbohydrate material has been fermented away. In these experiments a solution of pectin to which the usual inorganic salts had been added was sterilised before adjustment of the pH ¹. Calcium carbonate was added subsequently and inoculations made from broth. At the end of 14 days the cultures were acidified with acetic and hydrochloric acid to remove calcium carbonate. After neutralisation with NaOH and filtration, the liquid was acidified with acetic acid and heated with an excess of calcium chloride. The calcium pectate was filtered off, washed and weighed. In Table IV is given the pectin remaining in a series of tubes, the pectin being expressed as calcium pectate.

Table V.

*Mg. of pectin remaining after fermentation at 30° C., for 14 days,
solution initially contained 327 mg.*

Organism		Organism		Organism	
1	0	8	92	12	242
2	0	9	20	13	85
3	0	10	88	16	6
5	124	11	100	17	82

It will be seen that there is considerable variation in pectin-decomposing ability, but this does not necessarily imply that there is great variation in the actual quantity of carbohydrate fermented, as it is only the intact pectin ring which is being estimated.

These fermentation tests having shown that the activity of the organisms isolated is not very great in pure culture and on purified substrates, it was thought worth while to test their action on straw. 15 gm. portions were autoclaved in small flasks, and 50 mg. nitrogen added to each in the form of a sterile solution of peptone or ammonium phosphate.

¹ The natural pH of pectin solution is about 4.0. If this be adjusted to 7.0 or thereabouts before sterilisation, a very considerable loss will occur. A pectin solution brought to $pH = 7.2$ and autoclaved for 15 minutes at 121° C. yielded only 59 per cent. calcium pectate.

The inoculations were made by adding 10 c.c. from a glucose-soil extract broth in which the organisms were actively growing, and in addition 85 c.c. water was added. After incubation at 30° C. for 1 month the total loss was determined. Inasmuch as this was in all cases small, no analyses for constituents were made.

Table VI.

*Fermentation of oat straw with various organisms at 30° C.
Loss per cent. of straw in one month.*

N source	Organism					
	1	7	8	12	16	17
Peptone	5.0	—	4.1	3.0	5.2	5.7
Ammonium phosphate	—	0.5	5.3	2.5	4.9	0.9

In the same period a mixed inoculum from an enrichment culture in hemicellulose medium caused a loss of 14.5 per cent. of the straw when supplied with organic nitrogen. It is clear that the type of organism isolated is not capable alone of bringing about any general decomposition of a plant material such as straw, or even of fermenting to any considerable extent the 20–30 per cent. of hemicellulose contained in straw.

III. DISCUSSION.

The purpose of the work described in the preceding pages was, by isolating some of the responsible organisms, and testing others, to account for the observed phenomenon that hemicelluloses are rapidly and often almost completely removed in the natural decomposition of plant materials under aerobic conditions. That some fungi in pure culture can achieve such a removal has been demonstrated unquestionably by Waksman(6) and Norman(3). The latter showed that some common *Aspergilli* would remove in pure culture practically as much hemicellulose as a mixed natural flora in the same time. Many of those fungi that have been isolated from such decompositions attack cellulose also, and do not seem, therefore, to be particularly specific to any one plant constituent.

Nevertheless it was not anticipated that all of the fungi tested would be able to utilise a purified hemicellulose as the sole carbohydrate source. Certain of the organisms chosen are not normally found in the soil. It would seem that the ability to utilise hemicellulose is widely distributed and common amongst fungi. The test, as made, was purely qualitative and gave no indication as to the extent or rate of utilisation. Nevertheless, in many cases there was a very obvious production of an exo-enzyme

472 *The Biological Decomposition of Plant Materials*

hydrolysing hemicellulose, and therefore a strong probability that fermentation would be rapid.

In the case of bacteria the situation is rather different, for no organisms have been described which in pure culture can validly be described as very active in decomposing this group of polysaccharides. Of the large number of aerobic organisms isolated by Waksman and Diehm⁽⁹⁾ none completely fermented the polysaccharide preparations upon which they were tested, and in the case of xylan, which corresponds most closely to the plant polyuronides here considered, only three organisms produced as much as 50 per cent. fermentation of a dilute solution (0.15 per cent.) in 6 weeks. This cannot be regarded as a rapid utilisation, nor, on this evidence, is it likely that such organisms play any important part in a natural decomposition in which the hemicelluloses may be more or less completely removed in a few days.

In all, it appears that while in soil and in manure there are bacteria of various types which are capable of attacking the hemicelluloses, they are relatively feeble in fermentative power, at least when tested on the purified polysaccharides. Furthermore, some of the organisms are not really active in utilising common sugars, producing little of either acid or gas. There is the possibility that the purified polysaccharide may be less available than the natural material, as has been suggested by Waksman and Diehm⁽⁹⁾. This raises again the question as to whether or not availability tests are not too drastic. Cellulose or starch decomposition is determined by giving the organism cellulose or starch as the sole source of energy. It has been shown that this is sometimes too rigid a condition, and that certain organisms when actively developing on a particular energy source, can, at the same time, cause decomposition of another substance upon which they are unable to develop alone. This is particularly true of certain fungi which will not grow upon cellulose agar plates, but which can be shown by analysis to be excellent decomposers of cellulose when growing upon a material such as straw. Similarly Shrikhande⁽⁵⁾ recently demonstrated that *Mycobacterium agreste* which had not been known to attack cellulose, or grow on cellulose plates, does in fact produce a very considerable decomposition of the cellulose of straw, and is a more active cellulose decomposer under these conditions than the well known organism *Spirochaeta cytophaga* (*Cytophaga Hutchinsoni*). Nevertheless there is evidence in the case of some of the organisms isolated in this work, and some of those obtained by Waksman and Diehm⁽⁹⁾ that the test of availability as applied on the purified hemicellulose is not too rigid, since when inoculated on to natural

materials, such as corn cobs or straw, they were not appreciably or considerably more active.

There is the further possibility that these organisms may be more effective when working in association, and it is unquestionably time that crude enrichment cultures produce a more rapid decomposition than do any of the organisms alone. This may be due to the presence of secondary organisms utilising sugars or other degradation products produced by the organism actually attacking the hemicellulose. Whether that is so must be decided by further investigation, but the fact remains that a search for bacteria in soil and manure which can actively attack and decompose the polyuronide type of hemicellulose has not been successful, though a number of organisms have been obtained which are rather feeble in their action in pure culture. These organisms are of common soil types, but can utilise an unusually wide assortment of sugars and polysaccharides. Indeed no sugar was found to be unavailable, and differences were only observed by supplying to them sugar acids not likely to occur naturally.

Considering therefore this problem in its wider aspects, it seems probable that the ability to ferment hemicelluloses will be found to be a general property of common soil fungi, in contrast to the rather restricted fermentation by bacteria, and that to the fungi must be ascribed a prominent rôle in the earlier stages of the decomposition of plant materials containing hemicelluloses.

IV. SUMMARY.

1. The decomposition of the hemicelluloses of plant materials occurs rapidly under normal aerobic conditions. Since a number of fungi have been shown by analyses of pure culture rots to be active in fermenting this group, as well as cellulose, some seventy common fungi, mostly *Aspergilli* and *Penicillia*, were tested as to their ability to utilise on agar plates the crude hemicellulose from oat straw.

2. All of the fungi tested grew reasonably well on the hemicellulose medium, the majority at least as well as on glucose, and a number better than on that sugar. In some cases there was production of a cleared zone round the colony due to an *exo-enzyme*. It is likely that ability to ferment hemicelluloses will be found to be a general property of common fungi.

3. From soil, manure, and straw, twenty aerobic bacteria were isolated capable of utilising hemicelluloses. Three of these were thermophilic

474 *The Biological Decomposition of Plant Materials*

forms. Their morphological characters and biochemical reactions were determined.

4. All the organisms utilised a very wide range of sugars and polysaccharides; one group produced some acid and gas, the remainder little. Differentiation was obtained on saccharic acid and certain disaccharide acids.

5. Five of the mesophilic organisms agreed closely with the description of *Achromobacter ubiquitum* (Jordan). The remainder were aerobic spore-formers, though not all Gram-positive. The negative forms may represent new species.

6. None of the organisms in pure culture could validly be described as active in decomposing the isolated hemicellulose, or that *in situ* in straw.

7. Fungi seem to be more active and important in the natural decomposition of hemicelluloses than bacteria, unless when working in association the rather restricted individual fermentative powers of the latter are considerably enhanced.

V. ACKNOWLEDGMENTS.

The work described was largely carried out during 1931 in the Department of Agricultural Bacteriology, University of Wisconsin. The author is much indebted to Prof. Hastings for providing facilities in that Department, and to Dr E. B. Fred and Dr W. H. Peterson for their constant assistance and ready advice.

REFERENCES.

- (1) HAWLEY, L. F. and NORMAN, A. G. (1932). The differentiation of hemicelluloses. *Ind. and Eng. Chem.* XXIV, 1190-5.
- (2) JORDAN. (1890). *Report Mass. State Board of Health*, p. 830.
- (3) NORMAN, A. G. (1931). The biological decomposition of plant materials. IV. The biochemical activities on straw of some cellulose-decomposing fungi. *Ann. App. Biol.* XVIII, 244-59.
- (4) REGE, R. D. (1927). The decomposition of cellulosic materials. *Ibid.* xiv, 1.
- (5) SHRIKHANDE, J. G. (1933). The production of mucus during the decomposition of plant materials. II. The effect of changes in the flora. *Biochem. Journ.* XXVII, 1563-74.
- (6) WAKSMAN, S. A. (1931). Decomposition of the various chemical constituents of fresh plant materials by pure cultures of fungi and bacteria. *Arch. Microbiol.* II, 136-54.

- (7) WAKSMAN, S. A. and DIEHM, R. A. (1931). On the decomposition of hemicelluloses by micro-organisms. I. Nature, occurrence, preparation, and decomposition of hemicelluloses. *Soil Sci.* xxxii, 73-95.
- (8) ——— (1931). On the decomposition of hemicelluloses by micro-organisms. II. Decomposition of hemicelluloses by fungi and actinomycetes. *Ibid.* xxxii, 96-117.
- (9) ——— (1931). On the decomposition of hemicelluloses by micro-organisms. III. Decomposition of hemicelluloses by aerobic and anaerobic bacteria. *Ibid.* xxxii, 119-39.

(Received April 2nd, 1934.)

[FROM THE BIOCHEMICAL JOURNAL, Vol. XXIX, No. 1 pp. 116-132, 1935]

[*All Rights reserved*]

XVI. THE BIOLOGICAL OXIDATION OF CARBOHYDRATES.

IV. THE PHOSPHORUS REQUIREMENTS OF PERCOLATING FILTERS.

By SAMUEL HARRY JENKINS.

*From the Fermentation Department, Rothamsted Experimental
Station, Harpenden.*

(Received November 3rd, 1934.)

THE purification of sewage and certain trade effluents can be effected in percolating filters by allowing the polluting liquids to trickle through a well-aerated bed of material such as clinker, gravel *etc.*, suitably graded as regards size of particles. The first stage in the purification may be the physical retention of some of the polluting matter within the filter, but ultimately purification depends upon the breakdown and oxidation by living agencies of dissolved or precipitated organic matter. Although the biological nature of the process has long been recognised, insufficient attention has been given to a study of the conditions necessary to maintain the organisms responsible for purification in a state of maximum activity. A study of this kind should include experiments on the effect of changes in concentration of compounds of nitrogen, phosphorus and potassium on the rate of oxidation of the organic matter. Sewages of domestic origin are invariably rich in nitrogenous compounds, while they also contain fats, carbohydrates and salts of potassium and phosphorus. It is improbable, therefore, that the organisms responsible for the oxidation of domestic sewage liquors will be without any essential food material. Some waste waters containing organic matter, such as those from beet-sugar factories, may, however, have insufficient quantities of certain materials to ensure optimum rate of purification in percolating filters.

In a study of the process of biological oxidation of effluents from beet-sugar factories, Richards and Cutler [1933] have shown that the rate of oxidation of the carbohydrates in solution is influenced by the amount of available nitrogen. In view of the lack of data regarding the relationship between the supply of compounds of phosphorus and the rate of oxidation of organic matter, particularly carbohydrates, in percolating filters an investigation has been made with the objects of examining (1) the effect of phosphate on the rate and extent of oxidation of sucrose; (2) the relationship between the quantity of phosphate and the removal of nitrogen from solution; (3) the availability to micro-organisms of organic compounds of phosphorus; (4) the influence of phosphate supply on the development of an active biological film on the filter media. The results are described in the present paper. Similar work published by Barritt [1931] while these experiments were in progress will be referred to later.

EXPERIMENTAL.

Filters constructed from glass tubes containing glass medium made from broken bottles were used for the first set of tests and the results are described in Exp. 1. After the filters had been supplied for 28 days with solution con-

taining 0.1 % sucrose, pools of liquid formed on the surface of one filter owing to a heavy growth of film in the upper portion. Choking was avoided in a second series of experiments by using glass tubes as medium instead of broken glass. Details of construction of the filters have already been described [Jenkins, 1933, 1]. The results of this second set of tests are described as Exp. 2. In Exp. 3 the results are given of a large-scale trial at a beet-sugar factory. The main purpose of the experiment was to test the effect of inoculating selected organisms on to a filter. The liquids filtered were effluents from the beet-pulp presses of the factory after treatment in a sedimentation tank to remove suspended solid matter. During the period of sedimentation some fermentation occurred and the liquids became acid.

Method of analysis. Sugars, nitrogen as ammonia, nitrite and nitrate were determined by methods described previously [Jenkins, 1933, 2]. Phosphorus in the inorganic and organic forms was determined colorimetrically by the Denigès method as modified by Parker and Fudge [1927]. In the determination of phosphorus in organic combination, however, 1 ml. of 10 % calcium acetate solution was used instead of $\text{Mg}(\text{NO}_3)_2$ for igniting with the residue left after evaporation to dryness.

Experiment 1.

Each filter consisted of four glass cylinders with perforated metal plates at the lower ends. The sections were joined together by rubber bands and could easily be disconnected when samples of the effluent from any of the upper sections were required. The filtering medium was broken glass graded $\frac{1}{16}$ – $\frac{1}{4}$ in. Solutions of nutrient substances in the concentrations given below were filtered at a rate of 2 litres per day (100 gallons per cubic yard of filtering medium per day, abbreviated g.y.d.). These solutions each contained 5 parts N per 100,000 and

Solutions filtered from beginning of experiment until the 22nd day.

Filter ...	A_1 g.	B_1 g.	C_1 g.
Sucrose	1.000	1.000	1.000
NH_4Cl	0.191	0.191	0.191
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.050	0.050	0.050
$\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$	0.050	0.050	0.050
NaCl	0.005	0.005	0.005
K_2HPO_4	Nil	0.00736	0.0736
Fe_2Cl_6	Trace	Trace	Trace
Distilled water to	1 litre	1 litre	1 litre

the concentrations of P_2O_5 were 0, 0.3 and 3.0 parts per 100,000 to filters A_1 , B_1 and C_1 , respectively. From the 23rd day until the end of the experiment on the 150th day 0.05 g. K_2SO_4 per litre was added to the solution to ensure an excess of potassium. Ammonium bicarbonate replaced ammonium chloride as the source of nitrogen from the 68th day until the end of the experiment for a reason given later, but the amount of N added remained unchanged.

The results of the experiment, showing the amounts of sucrose, P_2O_5 and N left in the effluents are given in Table I.

Oxidation of sugar. A small proportion of the sugar in the solution fed to filter A_1 appeared to be oxidised during passage through the filter, but the amount did not exceed 18 % of the sugar in the nutrient solution. The filtering medium was apparently devoid of organic film but microbiological examinations made by Miss Dixon proved that a few protozoa were present. It is concluded

Table I. *Effect of phosphate on biological oxidation of sucrose and removal of nitrogen.**Exp. 1. Glass filters and glass medium ($\frac{1}{16}$ in. to $\frac{1}{2}$ in. graded glass).*Filter A_1 received sucrose equivalent to 105.3 parts glucose per 100,000 of solution, and no P_2O_5 .Filter B_1 received the same amount of sucrose as A_1 , and 0.3 part P_2O_5 per 100,000.Filter C_1 received the same amount of sucrose as A_1 , and 3.0 parts P_2O_5 per 100,000.

Day of exp.	Parts per 100,000.		Sucrose in effluent from filter calculated as glucose. Amount supplied, 105.3 parts			Nitrogen in effluent from filter. N supplied, 5 parts		
	P_2O_5 in effluent from filter							
	B_1 (0.3 supplied)	C_1 (3.0 supplied)	A_1	B_1	C_1	A_1	B_1	C_1
14	0.0	1.25	86.0	3.6	0.4	4.800	3.530	1.430
19	0.012	1.50	95.9	19.6	1.9	4.809	3.907	3.170
27	0.0	2.50	—	0.9	1.1	4.539	3.998	4.556
36	0.002	1.50	100.4	2.6	0.7	4.916	3.041	1.176
43	0.0	1.39	—	2.3	0.3	4.460	3.442	2.675

therefore that in filter A_1 the absence of phosphate almost inhibited the growth of film so that not more than 18 % of a 0.1 % solution of sugar could be oxidised.

The addition of 0.3 part P_2O_5 per 100,000 as potassium phosphate to filter B_1 had a marked effect on the amount of sugar oxidised. When the first analyses were made after the filters had been in operation for 13 days 96.6 % of the sugar was being oxidised and over the first 43 days of the experiment the average amount decomposed was 94.5 %. Although the percentage oxidation of sugar was high the amount of film in B_1 was not considerable and consisted of a thin slime covering the glass medium in the uppermost sections, with much less in the three lower sections. The greatest contribution towards the total amount of sugar oxidised was made by section 1, which alone accounted for 89.7 % out of a total average oxidation of 94.5 %.

Filter C_1 received 3 parts P_2O_5 per 100,000 as K_2HPO_4 and gave a daily average oxidation of sugar of 99.2 % over the first 43 days. It should be pointed out that differences of a few per cent. are of greater significance when the purification figure approaches 100 % than at, say, 50. The amount of work, measured by extra filter space, required to increase a percentage purification from 50 to 60 is much less than that required to produce the same increment from 90 to 100. Thus section 1 of C_1 oxidised 98 % of the total sugar but a small amount of sugar was still present in the final effluent.

In filter C_1 the growth of film covered the surface of the medium and by the end of the first month the solution dropping on to the filter did not pass through immediately but gathered in pools on the surface and drained through the filter at a slower rate than was desired. This condition of ponding led to the initiation of the second series of experiments, the results of which are described later.

Removal of phosphorus and nitrogen. When no phosphate was supplied the amount of sugar removed from solution was, as previously stated, small and the amount of nitrogen removed was also small. Out of a total of 5 parts N supplied as NH_4Cl an average of only 0.3 part was taken up over a period of 43 days (Table I). An addition of 0.3 part P_2O_5 to B_1 increased the average removal of N over the 43 days to 1.416 parts. The quantity of P_2O_5 added in this case apparently did not provide enough phosphorus to cause all the dissolved sugar to be oxidised since some sugar remained after all the phosphorus

had been removed from solution. Not more than a trace of phosphorus was ever found in the final effluent from B_1 . The figures obtained with filter C_1 , however, showed that 3 parts P_2O_5 per 100,000 provided more than enough phosphorus. Practically all the sugar supplied was oxidised while only 1.63 parts of the P_2O_5 were removed. The fall in concentration of nitrogen in C_1 was 2.4 parts from an initial supply of 5 parts N.

Although it seemed unlikely that intermediate compounds such as hexosephosphate would be present in the effluents, as these substances do not ordinarily pass out of the living cell into the substrate, tests were made for the presence of soluble organic compounds of phosphorus in the effluents from the filters. The effluents were analysed for inorganic and organic forms of phosphorus as soon as sufficiently large volumes had been collected. In consequence the solutions were on an average 2 hours old at the time of analysis. The p_H values of the effluents of filters B_1 and C_1 were about 3.0. Under these conditions soluble organic compounds of phosphorus were invariably absent, although it is almost certain that at times organic phosphorus would be contained in the small amounts of film in suspension occasionally washed out of the filters. The effluents were settled for a few minutes so as to exclude visible suspended matter from the samples used for the determination of phosphorus.

In order to determine the amount of organic phosphorus which could be recovered in a typical hexosephosphate by the Parker and Fudge modification [1927] of the Denigès method, an examination was made of a sample of barium hexosephosphate, kindly supplied by Prof. A. Harden. The preparation contained 9.5 % moisture and the phosphorus content of the dry substance was approximately: total P_2O_5 , 22.7 %; organic P_2O_5 , 20.6 %; inorganic P_2O_5 , 2.1 %. Solutions of 10 mg. of the barium compound per litre were made up so that there were present: total P_2O_5 , 2.05 parts per million; organic, 1.87 parts; inorganic, 0.18 part. The corresponding figures as determined colorimetrically by the modified Denigès method were 2.1, 1.9, and 0.2 respectively, showing that it was reliable for the determination of the organic phosphorus in hexosephosphates. The p_H value of the distilled water in which the solutions were made up varied between 5.5 and 6.0. After storage for several hours it was observed that the amount of inorganic phosphorus in the hexosephosphate solutions had increased. Thus on one occasion 0.86 part inorganic P_2O_5 per million was found after the solution had stood for 8 hours, while on another occasion all the hexosephosphate had been completely hydrolysed after standing 24 hours at room temperature. It seems that very dilute aqueous solutions of hexosephosphate undergo hydrolysis on storage; the solutions were made up in distilled water but no precautions were taken to ensure that they were sterile. An alternative colorimetric method described by Zinzadé [1932] was tried, since the colour standards are more permanent than in the Denigès method, but the hexosephosphate appeared to be hydrolysed to inorganic phosphorus during analysis, owing to the greater concentration of mineral acid used by Zinzadé.

It has already been mentioned that the p_H values of the final effluents from filters B_1 and C_1 were about 3.0. When the source of nitrogen added was changed on the 68th day from ammonium chloride to ammonium bicarbonate, the p_H values of the solutions were either unchanged or increased on filtration through B_1 and C_1 . The p_H values of the nutrient solutions supplied to the filters were 5.5 for B_1 and about 6.0 for C_1 . Barritt [1931] filtered 0.2 % solutions of sucrose *plus* ammonium sulphate through sectional filters and found that the p_H of the liquid changed from an initial value of 7.0 to 5.5 in the

second upper section and to 7.1 in the sixth and final section; the reduction and subsequent increase in p_H were attributed to the production and oxidation of organic acids. The following considerations indicate that the observed differences in the p_H values of the solutions supplied to the filter and of the final effluents in Exp. 1 (Table II) were due mainly to the removal of nitrogen from

Table II. *Effect of source of nitrogen on formation of acid.*

Filters fed with sucrose solution and N as NH_4Cl (made up in distilled water) from start of experiment until the 68th day: after this they received N as NH_4HCO_3 .

A_1 received no P_2O_5 .

B_1 received 0.3 part P_2O_5 per 100,000.

C_1 received 3.0 parts P_2O_5 per 100,000.

N removed expressed as parts per 100,000.

Day of exp.	Nutrient solution p_H	1st section		2nd section		3rd section		4th section		Filter
		p_H	N re-moved	p_H	N re-moved	p_H	N re-moved	p_H	N re-moved	
14	5.4	5.1	—	5.1	—	4.8	—	4.6	0.2	A_1
	5.8	3.6	—	3.2	—	2.9	—	2.9	1.47	B_1
	6.6	2.9	—	2.9	—	2.9	—	2.9	3.57	C_1
19	5.6	—	—	—	—	—	—	4.4	0.19	A_1
	5.4	—	—	—	—	—	—	3.2	1.09	B_1
	6.0	—	—	—	—	—	—	3.0	1.83	C_1
26	5.4	5.2	0.37	5.2	0.00	5.4	0.00	5.6	0.00	A_1
	5.0	3.0	1.59	3.0	0.08	3.0	0.00	3.0	0.34	B_1
	4.7	—	3.38	3.0	0.08	3.0	0.09	3.0	0.28	C_1
43	6.0	6.0	0.56	5.1	0.04	5.3	0.49	5.5	0.38	A_1
	5.6	3.0	1.54	3.2	0.00	3.2	0.00	3.3	0.89	B_1
	6.2	3.2	2.07	3.2	0.00	3.1	0.34	3.1	1.02	C_1
70	7.4	—	—	—	—	—	—	7.2	—	A_1
	7.3	—	—	—	—	—	—	6.9	—	B_1
	7.1	—	—	—	—	—	—	6.0	—	C_1
75	7.4	—	—	—	—	—	—	7.3	—	A_1
	7.3	—	—	—	—	—	—	6.8	—	B_1
	7.1	—	—	—	—	—	—	6.4	—	C_1

ammonium chloride and the liberation of an equivalent quantity of free HCl. This was not neutralised or absorbed by the glass medium and therefore passed out with the effluent. For example, on the 19th day of the experiment the amounts of nitrogen expressed in parts per 100,000 removed by filters A_1 , B_1 and C_1 were 0.19, 1.09 and 1.83 respectively. Assuming that equivalent amounts of free HCl were liberated, the strength of acid in the effluents would be 0.137, 0.781 and 1.308 $N/1000$ for A_1 , B_1 and C_1 respectively. Sufficient acid would thus be produced to cause the drop in p_H value from 5.6 in the nutrient solution to 5.0 in the final effluent of A_1 , from 5.4 to 3.2 in B_1 and from 6.0 to 2.8 in C_1 . If chlorine ions, equivalent to the nitrogen assimilated, were taken up by the filter this argument would be untenable. Determinations of Cl were made therefore with silver nitrate and the following results were obtained:

Cl in nutrient solutions and effluents from filters.

Parts per 100,000.

	Filter ...	A_1	B_1	C_1
Cl in nutrient solution	...	14.29	14.29	14.29
" effluent of section 1	1	14.26	14.26	14.87
" " 2	2	14.26	14.57	14.57
" " 3	3	14.27	14.72	14.72
" " 4	4	15.17	15.02	14.26

These results show that there was no uptake of Cl by the filters commensurate with the removal of nitrogen. If the decreases in p_H recorded with filters B_1 and C_1 were caused entirely by organic acids ionised to the same degree as acetic acid, then the concentration of acid required would be greater than 0.1 %. As the original amount of sugar was only 0.1 % a larger quantity of acetic acid could not be present. Moreover when determinations of the relative amounts of organic matter in the nutrient solutions and in the effluents were made by a test in which both sugar and organic acids are oxidised, *viz.* the biochemical oxygen demand, known as the "Dissolved oxygen absorbed test" in the Ministry of Health Methods [1929], results as follows were obtained:

Biochemical oxygen demand (B.O.D.) in 5 and 20 days.

Average results of five analyses made at different times on the liquids filtered and the effluents from the sections.

Parts per 100,000.

Filter	Crude liquor filtered		Effluent of section 1		Effluent of section 2		Effluent of section 3		Effluent of section 4	
	5 20		5 20		5 20		5 20		5 20	
	days	days	days	days	days	days	days	days	days	days
A_1	69.2	103.6	71.1	93.6	63.4	78.7	61.1	99.4	56.3	95.3
B_1	64.1	105.3	46.2	73.2	39.1	55.0	45.1	69.2	33.1	55.4
C_1	69.6	96.2	26.4	41.9	25.4	45.1	15.4	28.4	8.6	18.3

A 0.1 % solution of sugar, acetic or lactic acid has a maximum B.O.D. figure of about 112, which is approached in 20 days. Even if the whole of the B.O.D. were due to one of these organic acids there would still be insufficient acid to account for the observed fall in p_H value during filtration of the sugar solutions. This evidence indicates that the differences in p_H values of the original solution and filter effluents are caused by mineral acid set free from the ammonium salt, a conclusion not necessarily conflicting with that of Barritt, if the differences between the two experimental conditions are considered. The 0.2 % sugar which Barritt used may have given a growth of film in which acid production proceeded more quickly than oxidation of sugar and acid.

Experiment 2.

This experiment was undertaken because (a) two of the filters used in Exp. 1 showed signs of choking before the completion of the work, (b) the development of highly acid effluents consequent on the use of ammonium chloride as a source of nitrogen may have interfered with the development of organic film and nitrification processes. To provide a larger air space in the glass filters used for Exp. 2 the broken glass medium was replaced by tubes, 1 cm. diameter and 1.5 cm. long. The sectional filters employed were A_2 , B_2 and C_2 and they received solutions of the same composition as were supplied to A_1 , B_1 and C_1 respectively in Exp. 1, except that (1) nitrogen was supplied as ammonium bicarbonate, (2) the nutrient solution filtered in A_2 contained 0.05 g. K_2SO_4 per litre. A rate of flow of 2 litres per day, equivalent to 100 g.y.d., was maintained and the experiment was continued for 73 days.

Development of film. At the end of the experimental period of 73 days there was no film visible on any of the glass tubes in filter A_2 ; the filter did not appear to retain liquid as long as B_2 or C_2 . The film in B_2 increased slowly in amount as the filter aged and apparently had not reached maximum activity at the

termination of the experiment. Filter C_2 had a white gelatinous growth on the surface within a fortnight and after this the film spread downwards through the first and second sections. At the end of the experiment the first section had a thick, pale yellow, gelatinous film but it was not choked, and liquid could be filtered at twice the desired rate without surface ponding. Section 2 in filter C_2 contained much film, though this was less in amount than in section 1, while there was considerably less film in section 3 than in section 2. In section 4 the small quantity of film present consisted of a thin slime on the glass tubes. It was observed in the early stages of the experiment that much of the film in sections 2 and 3 was formed by growths in the section above being washed into the one below, forming centres of inoculation from which the film spread to other parts of the filter.

Table III. *Effect of supply of phosphate on sucrose oxidation and removal of nitrogen.*

Exp. 2. Glass filters and tubular glass medium: Rate of flow = 100 g.y.d.

Filter A_2 received sucrose equivalent to 105.3 parts glucose per 100,000 and no P_2O_5 .

Filter B_2 received sucrose equivalent to 105.3 parts glucose per 100,000 *plus* 0.3 part P_2O_5 .

Filter C_2 received sucrose equivalent to 105.3 parts glucose per 100,000 *plus* 3.0 parts P_2O_5 .

2 litres of nutrient solution supplied daily = 100 g.y.d.

Day of exp.	P_2O_5 in final effluent		Parts per 100,000.					
	B_2 (0.3 supplied)	C_2 (3.0 supplied)	Sugar in final effluents: glucose equivalent			Nitrogen in final effluents (5 parts N supplied)		
			A_2	B_2	C_2	A_2	B_2	C_2
26	0	2.00	96.8	67.1	2.0	4.98	4.07	2.20
33	0	—	107.0	61.5	2.0	—	—	—
44	—	1.00	111.8	58.0	0.4	3.73	4.13	2.67
58	0	0.75	108.5	50.3	0.8	4.79	3.75	2.98
60	0	1.00	103.2	57.8	0.9	4.81	3.98	2.36
66*	0.002	2.60	96.3	33.7	1.5	5.07	4.47	3.70
71	—	—	106.4	58.4	7.1	4.36	3.75	3.51
73	0	1.38	90.0	34.0	4.6	4.94	3.68	1.87

* Filter C_2 received 4 litres daily from 61st day.

Oxidation of sugar (Table III). The amounts of sugar oxidised in the three filters A_2 , B_2 and C_2 at different stages of the experiment are shown in Fig. 1.

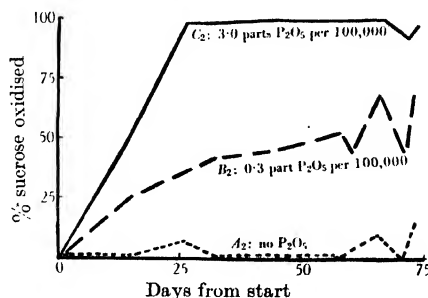


Fig. 1. Effect of phosphorus supplied on oxidation of sucrose

The curves indicate a definite relationship between the supply of phosphorus and the amounts of sugar oxidised, provided adequate additions of other essential

mineral salts are made to the nutrient solutions. In the absence of added phosphorus filter A_2 rarely oxidised more than 14 % of the sugar, and the average amount oxidised over a period of 73 days was only 2.6 %. Filter B_2 , however, with 0.3 part P_2O_5 per 100,000 decomposed 36 % of the carbohydrates after a period of 26 days. The percentage oxidation increased steadily to 50 after 50 days and on the 73rd day, at the end of the experiment, filter B_2 was oxidising 68 % of the sugar. The trend of the curve for B_2 suggests that the amount of sugar oxidised by this filter would have continued to increase if the experiment had been prolonged. The results show that the smallness of the amount of phosphorus supplied restricted the growth of a population sufficiently active to oxidise all the sugar. As the filter accumulated phosphorus and the film developed, the amount of organic matter oxidised increased. Two of the many factors which influence biological oxidation in percolating filters are thus seen to be the supply of phosphorus and the time factor. When the results obtained with filters B_1 and B_2 are compared a striking difference is observed in the amounts of sugar oxidised. After receiving 0.1 % solution of sugar daily for 43 days (Table I) filter B_1 oxidised 98 % daily whereas B_2 oxidised only 43 % after operating for the same period (Fig. 1). This comparison emphasises another important factor in filtration, *viz.* the relationship between the size of filtering medium and the efficiency of the filter. Apart from the change made in the source of nitrogen the only difference between B_1 and B_2 was that B_1 contained small pieces of solid glass while B_2 was filled with larger pieces of tubular glass. This difference in size of medium affects the phosphorus requirements of the filters in the following manner. Sufficient phosphorus must be provided to B_1 to build a film able to retain the solution passing through the filter long enough for the sugar to be decomposed. With the small grade of medium in B_1 a limited growth of organisms was sufficient to join up the particles of medium and so increase the time of contact of the filter. The quantity of phosphorus used for B_1 , when supplied to B_2 , gave about the same growth, but here the film only covered the surface of the glass tubes and did not join them together so as to make a continuous growth. Spaces were thus left in the filter through which sugar passed and escaped oxidation. With filter C_2 , which received 3 parts P_2O_5 per 100,000, the amount of sugar decomposed reached 98 % in the first 26 days and later continued to improve until the oxidation attained was almost quantitative. Even when the rate of flow was doubled on the 60th day of the experiment over 93 % of the carbohydrate underwent oxidation.

The effect of supply of phosphorus on the contribution made by the different parts of the filter towards the total oxidation of the carbohydrate was found by dismantling the filters at various intervals and analysing the effluents of the different sections of the three filters. The results appear in Table IV. The samples were taken successively from the different sections over a period of 16 hours, beginning at section 4 and working upwards. Consequently the samples did not correspond exactly, and probably for this reason, the analytical results do not always show a regular decrease in sugar content from the inlet to the outlet of the filter. The figures in Table IV show that the effect of withholding phosphate from filter A_2 was to inhibit inversion and oxidation of sugar. Traces of invert sugar were generally present in the effluents from the first section of A_2 and in these cases the same quantity persisted almost unchanged in the effluents from lower sections. These small amounts of invert sugar may have been due to analytical errors in the Hagedorn-Jensen method as carried out by the author [Jenkins, 1933, 2]. With filter B_2 a high proportion of invert sugar always appeared in the effluent from section 1 with a tendency for the

Table IV. *Experiments with glass filters filled with tubular glass medium.*Solution fed to filter A_2 : Sucrose equal to 105.3 parts glucose; 5 parts N as NH_4HCO_3 ; no P_2O_5 .Solution fed to filter B_2 : Sucrose equal to 105.3 parts glucose; 5 parts N as NH_4HCO_3 ; 0.3 part P_2O_5 ; per 100,000.Solution fed to filter C_2 : Sucrose equal to 105.3 parts glucose; 5 parts N as NH_4HCO_3 ; 3.0 parts P_2O_5 ; per 100,000.

Total sugar and invert sugar (given as glucose) in effluents from sections.

Parts per 100,000.

Day of exp.	Section 1		Section 2		Section 3		Section 4	
	Total sugar	Invert sugar	Total sugar	Invert sugar	Total sugar	Invert sugar	Total sugar	Invert sugar
Filter A_2 . 2 litres solution fed daily.								
15	93.2	3.1	90.4	3.0	88.8	2.8	88.2	2.8
26	103.5	0.5	101.5	0.8	100.3	0.5	96.8	0.4
30	—	—	—	—	—	—	—	—
33	—	—	—	—	—	—	107.0	0.7
44	110.3	0.5	111.8	0.5	111.8	0.8	111.8	0.8
58	—	—	—	—	—	—	108.5	1.2
60	101.0	1.0	107.2	1.0	103.2	1.0	—	—
66	—	—	—	—	—	—	96.3	1.5
71	—	—	—	—	—	—	106.4	3.3
73	—	—	—	—	—	—	90.0	1.7
Filter B_2 . 2 litres solution fed daily.								
15	81.2	17.1	63.5	16.2	69.7	15.8	65.0	14.8
26	59.2	20.9	89.3	35.4	78.0	36.4	67.1	30.7
30	—	—	—	—	—	—	—	—
33	—	—	—	—	—	—	61.5	56.4
44	74.0	25.2	80.0	30.1	65.0	37.3	58.0	50.1
58	—	—	—	—	—	—	50.3	26.0
60	49.0	23.4	58.0	24.8	48.7	27.9	57.8	33.7
66	—	—	—	—	—	—	33.7	24.0
71	—	—	—	—	—	—	58.4	26.6
73	—	—	—	—	—	—	34.0	25.3
Filter C_2 . 2 litres solution fed daily until 60th day; after that 4 litres fed daily.								
15	44.5	16.8	48.9	14.2	46.4	14.6	40.6	12.9
26	22.1	6.8	5.8	4.0	1.4	1.0	2.0	0.6
30	44.4	26.1	31.9	25.4	29.1	20.0	18.7	18.7
33	—	—	—	—	—	—	2.0	2.0
44	3.5	1.6	1.2	0.7	0.9	0.3	0.4	0.3
58	—	—	—	—	—	—	0.8	0.8
60	3.4	0.8	0.8	0.4	0.3	0.3	0.9	0.6
66	—	—	—	—	—	—	1.5	1.5
71	—	—	—	—	—	—	7.1	3.2
73	—	—	—	—	—	—	4.6	1.1

amount of invert sugar to increase on passage down the filter. On two occasions the sugar in the final effluent was largely the inverted product, there being 56.4 parts invert sugar in a total sugar content of 61.5 parts on the 33rd day and 50.1 out of a total of 58.0 on the 44th day. The view has previously been advanced [Jenkins, 1933, 2] that the occurrence of a large proportion of invert sugar in the effluents of a filter receiving solutions of sucrose is a sign of an immature film, and that increase in efficiency of oxidation brings about a decrease in the proportion of invert sugar in the total sugar. The observations made above on the composition of the effluents from the different sections support this view, since B_2 never reached maturity during the 73 days of the experiment. Although the film in B_2 was unable to oxidise the carbohydrates that passed through it, the filter was yet able to invert a large proportion of

the sugar it received. The film in section 2 of filter B_2 was small in amount and considerably less in the two lower sections. All these sections however were able at times to bring about the inversion of appreciable quantities of sugar in the absence of measurable amounts of inorganic phosphate in the effluents (less than 0.002 P_2O_5 per 100,000) but were unable to carry the decomposition much beyond this stage. The addition of 3 parts P_2O_5 per 100,000 to the sugar solution fed to filter C_2 resulted in the growth of much film in section 1 and less in section 2. Only a very small amount of growth took place in sections 3 and 4, owing to the low concentration of sugar in the solutions reaching these sections. As previously observed, some of the film in section 2 was washed down from the upper part of the filter.

A large proportion of the sugar which passed through filter C_2 was hydrolysed to invert sugar: on the 30th day all the sugar contained in the effluent of section 4 appeared in this form. The concentration of sugar in effluent 4, filter C_2 , was negligible after the 30th day as long as the rate of 100 g.y.d. was maintained.

Phosphorus and nitrogen removed. The quantities of phosphorus and nitrogen taken up by the filters are given in Table V. The figures show that with one

Table V. *Phosphate and nitrogen in effluents at different levels of the filters.*

Exp. 2. Glass filters and tubular glass medium: rate of flow — 100 g.y.d.

Filter A_2 received sucrose equivalent to 105.3 parts glucose per 100,000 and no P_2O_5 .

Filter B_2 received sucrose equivalent to 105.3 parts glucose per 100,000 plus 0.3 part P_2O_5 .

Filter C_2 received sucrose equivalent to 105.3 parts glucose per 100,000 plus 3.0 parts P_2O_5 .

		Parts per 100,000.								
		26th day of Exp.				60th day of Exp.				
		P_2O_5		Nitrogen		P_2O_5		Nitrogen		
		B_2	C_2	B_2	C_2	B_2	C_2	A_2	B_2	C_2
Nutrient solution contained		0.3	3.00	5.00	5.00	0.30	3.00	5.00	5.00	5.00
Effluent of section 1 contained		Trace	2.00	4.04	2.23	0.00	1.00	4.92	4.36	3.40
"	2	0	1.87	4.07	2.09	0.00	0.76	4.81	4.28	2.88
"	3	0	1.50	4.07	1.92	0.00	0.88	4.81	4.17	2.77
"	4	0	2.00	4.07	2.20	0.00	1.00	4.81	3.98	2.36

exception the phosphate added to B_2 was removed quantitatively by this filter. Reference to Tables IV and V shows that section 1 of B_2 was responsible for the removal of the whole of the phosphate and for the oxidation of most of the sucrose. The absence of phosphorus from the solutions of sugar leaving this section accounts for the poor development of film in the remaining sections of this filter. Varying amounts of nitrogen were taken up by B_2 during the early stages of the experiment but later small amounts were removed continuously. An average of 1.02 parts N per 100,000 was removed from the solutions, which before filtration contained 5 parts. Table V shows that most of the nitrogen was absorbed by section 1 of B_2 and only small amounts by lower sections. The solutions leaving section 1 therefore contained sugar and ammonium salts but practically no phosphorus. On the other hand, filter C_2 received more than enough phosphorus for its requirements (Table V), an average of 1.54 parts P_2O_5 being taken from a supply of 3 parts. This uptake of phosphorus enabled C_2 to oxidise almost completely 2 litres of a solution of 0.1 % sucrose every day for the first 60 days and 4 litres daily after the 60th day of the experiment.

The average amounts of nitrogen retained by the filters are obtainable from the data given in Table V and show that the uptake of this element depends upon the quantity of phosphorus available for the growth of the film. Thus in the absence of phosphorus only 6.6 % of the N supplied to A_2 was removed: B_2 and C_2 , which received 0.3 part and 3 parts P_2O_5 respectively, took up 20.5 and 44.9 % of N respectively.

Figures for the total amounts of C, N and P removed from solution by filters B_2 and C_2 during the experiment are presented in Table VI. The difference

Table VI. *Carbon, nitrogen and phosphorus removed from solutions.*

C in sugar solutions supplied to B_2 and C_2 = 42.1.					
N in sugar solutions supplied to B_2 and C_2 = 5.0.					
P in sugar solutions supplied to B_2 and C_2 = 0.13.					
Parts per 100,000.					
Filter B_2 Experimental period			Filter C_2 Experimental period		
First 44 days			Last 29 days		
C	N	P	C	N	P
17.2	1.83	0.13	23.4	1.07	0.13
Ratio C/N/P 9.4/1/0.071			21.9/1/0.12		
73 days					
C	N	P			
41.1	2.08	0.64			
Ratio C/N/P 19.8/1/0.31					

in the ratios of C, N and P in B_2 during the first 44 days and the last 29 days means that more carbon was oxidised for a given supply of N in the later stages of the experiment than in the earliest part. Although the C/N/P ratios for the last 29 days of B_2 and the whole period for C_2 are similar in respect of C and N yet the actual amounts of C and N oxidised and removed differed considerably in the two filters. The suggestion was made in a previous paper [Jenkins, 1933, 3] that nitrogen would be used most economically in a filter oxidising sugar if the initial C/N ratio were kept at about 30/1. A C/N ratio of 19.8/1 was obtained with C_2 because an excess of N was supplied. In the presence of less N the same amount of sugar would probably have been oxidised and C and N removed in a wider ratio. According to the figures given above, the minimum proportion of phosphorus required for the efficient utilisation of nitrogen would lie between 0.12 and 0.31 part available P (0.28 and 0.71 parts respectively P_2O_5) per part of nitrogen.

As previously mentioned, the formation of acid in percolating filters was found to arise from the use of NH_4Cl as the source of nitrogen. In Exp. 2 slight variations were found in the p_H values of the solutions undergoing filtration (Table VII) but reductions in p_H of the order observed when ammonium chloride

Table VII. *Average p_H values of nutrient solutions and effluent.*

Filter	Nutrient	Section effluents			
		1	2	3	4
A_2	7.8	7.8	7.8	7.9	7.8
B_2	7.3	7.2	7.3	7.3	7.1
C_2	7.1	7.6	7.2	7.3	7.0

was the source of N were not detected. There is thus no evidence that intermediate organic acids were formed to any considerable extent during the aerobic oxidation of 0.1 % sugar by the mixed flora of the percolating filter operated under the conditions observed in these experiments.

*Experiments 3 (a) and 3 (b).**The availability of compounds of phosphorus in percolating filters.*

Exp. 3 (a). The apparatus used for this experiment was the large scale filtration plant previously described by Richards and Cutler [1933]. The liquid treated by the biological filters was the effluent from the beet-pulp presses of a beet-sugar factory, diluted and partly fermented, so that it gave approximately the following results on analysis:

	Parts per 100,000
Oxygen absorbed in 4 hours from permanganate ...	122
Biochemical oxygen demand in 5 days	109
Biochemical oxygen demand in 20 days	156
Sucrose	68
Acetic acid	20
Lactic acid	20
Solids in suspension (4 % ash)... ..	42
P ₂ O ₅ , all organic	0.85
Nitrogen in organic combination	2.5
Nitrogen as ammonia	0.01

p_H, 5.1

The suspended matter consisted of cellulose and fibre derived from the sugar-beet root, while the organic nitrogen was mostly of protein origin and less readily available to micro-organisms than ammonia. The organic compounds of phosphorus were probably hexosephosphate, nucleoproteins and phospholipins derived from organisms and the cell contents of the beet. Liquid of the average composition given above was distributed at a rate of 50 g.y.d. over two filters. As the chief object of the experiment was to test the effect of inoculation on the rate of oxidation of the constituents of fermented press water, one of the filters was inoculated with organisms selected by Mr D. W. Cutler and Miss L. C. Crump, while the other filter was not inoculated. Despite this difference the final effluents of both filters were almost identical in chemical composition. Hence they have been considered as duplicates for the purpose of this experiment: the figures given are the averages of the analytical data from the inoculated and uninoculated filters. During the first 20 days the organic compounds of nitrogen and phosphorus present in the liquids filtered varied according to the composition of the factory press water, but known quantities of ammonia or potassium phosphate were added. The liquids filtered during this period could be classified as follows:

(1) For the first 7 days the liquids contained nitrogen as ammonia and in organic combination as well as 0.85 part P₂O₅ per 100,000 as organic phosphorus supplied by the pulp liquor.

(2) From the 8th day to 13th day the liquids contained nitrogen as ammonia and in organic combination besides phosphorus in organic combination and as added K₂HPO₄.

(3) From the 14th day to 18th day the substances in (2) were present, though the amounts of N and inorganic phosphorus were less.

(4) After the 20th day the liquids contained organic nitrogen, phosphorus in the organic and inorganic states and traces of ammonia.

The results of operating the filters under the conditions given above are summarised in Table VIII. The figures for percentage purification represent the averages of the amounts of oxidation of organic matter in the two filters as measured by the B.O.D. test. It must be pointed out that the conclusions

Table VIII. *Results of large-scale experiments.*

Day of Exp.	Rate g.y.d.	Parts per 100,000.							
		P ₂ O ₅ in liquid filtered		P ₂ O ₅ recovered in effluent.	N in liquid filtered		N recovered in effluent		Oxidation of organic matter %
		Inorganic	Organic. Average figure		Ammonia N	Organic N	Ammonia N	Organic N	
0-7		0	0.85	0	3.00	1.85	2.50	1.12	25
8	50 for 10 hrs. daily	3.0	"	0.30	3.00	1.85*	2.00	—	28
10		"	"	0.50	3.00*	2.48*	(Total N = 1.49)		55
11		"	"	0.50	3.00*	2.38*	(Total N = 1.03)		81
12		"	"	0.41	3.00*	1.60*	(Total N = 0.78)		75
13		"	"	0.56	2.5	3.76	0.07	1.16	86
14	50	1.5	"	0.38	1.5	3.34	0.05	1.49	86
15	50	"	"	0.02	2.25	2.00	0.03	1.21	85
16	50	"	"	0.02	1.00	2.16	0.01	1.25	78
18	100	"	"	0.22	—	—	—	—	66
20	100	"	"	0.75	0.08	2.80	0.03	—	51

* Approximate.

drawn should be regarded as tentative or as supporting those already given in the early part of the paper, for the reason that liquors of different composition were treated in the same filter in its early stages and not simultaneously in different filters. Hence comparisons are available only between the results of filtering liquids of different composition at different periods in a filter undergoing development.

If the results given above are considered with respect to the changes made in operating the filters the following conclusions may be drawn:

(1) *1st-7th days.* In the presence of organic phosphorus and nitrogen in organic and inorganic combination not more than 25 % of the organic matter was oxidised on filtration and only 17 and 39 % respectively of the inorganic and organic nitrogen supplied were removed by the filters. The amount of organic phosphorus removed from the liquid was not determined, but as an approximation it is assumed that the quantity was proportional to the uptake of organic nitrogen.

(2) *8th-13th days.* The response to the addition of an inorganic source of phosphorus on the 8th and on successive days was immediate. Inorganic phosphorus to the extent of 90 % was removed in the first 2 days and an average retention of 85 % was recorded over the period 8th to 13th days. The removal of inorganic nitrogen by the filters increased progressively from 33 % on the 8th day to 98 % on the 13th day, while the percentage purification increased from 28 to 86 in this period. This increase in percentage purification probably included the improvement in efficiency which generally accompanies the ageing or maturing of a filter. Cutler and Crump (unpublished work) found that the bulk of film rapidly increased in both filters between the 8th and 13th days.

(3) *14th-18th days.* A reduction in the amount of added phosphorus resulted in a greater percentage retention of P₂O₅, which increased to about 98. As long as ammonia was present, nitrogen in this form was removed almost quantitatively. The average oxidation of organic matter amounted to about 80 %.

(4) *20th day et seq.* When ammoniacal nitrogen was withheld the removal of inorganic phosphorus decreased and a rapid fall in the percentage oxidation followed. In the continued absence of inorganic nitrogen the percentage purification of organic matter remained at a low figure, in spite of the presence of an inorganic compound of phosphorus.

The figures above therefore show that (a) the phosphorus in the organic compounds contained in the press water of a sugar-beet factory is not so readily available to the micro-organisms in a percolating filter, even in the presence of sufficient available nitrogen, as an inorganic supply of phosphorus such as K_2HPO_4 ; (b) in the presence of potassium phosphate, nitrogen as ammonia is removed by the micro-organisms of a filter in preference to the organic compounds of nitrogen present in press water, with consequent improvement in the percentage purification of organic matter by the filter; (c) the removal of phosphorus as dissolved phosphate by a filter is slow and incomplete if the liquids filtered do not contain nitrogen in a form which can be rapidly utilised by the micro-organisms responsible for the activity of the filter. Press water appears to be deficient in this form of nitrogen.

Exp. 3 (b). The last conclusion, (c), received substantial corroboration from the results of an experiment briefly described below.

Two small laboratory filters were filled with gravel and supplied with solutions of the same composition as those used for Exp. 3 (a), at a rate of 100 g.y.d. Sufficient K_2HPO_4 was added to the solution fed to one filter to provide 1.5 parts P_2O_5 per 100,000; no such addition was made to the liquid supplied to the control filter. The crude liquor of both filters contained approximately 0.85 part P_2O_5 as organic phosphorus during the experimental period of 26 days. Analyses were made on 21 days and the following average results for percentage purification were obtained:

Average percentage purification of diluted press water.

Control filter received press water containing 0.85 part organic P_2O_5 per 100,000.

Phosphate filter received press water containing 0.85 part organic P_2O_5 and 1.5 parts inorganic P_2O_5 per 100,000 added as K_2HPO_4 .

Oxygen absorbed from N/8 KMnO ₄ % purification		B.O.D. in 5 days % purification		B.O.D. in 20 days % purification		% removal of total sugar	
Control	Phosphate	Control	Phosphate	Control	Phosphate	Control	Phosphate
46	52	44	38	36	37	53	37

In any one of the above tests the differences between the averages of the percentage purifications for the control and the phosphate filter are not significant.

The experiment therefore shows the oxidation of the organic matter in fermented press water by a percolating filter to be unaided by the addition of an available supply of phosphorus, if only those organic compounds of nitrogen normally found in press water are present.

DISCUSSION OF RESULTS.

The experiments described in this paper demonstrate that the influence of phosphorus on the rate of oxidation by a percolating filter of carbohydrates and the organic matter present in the press water of a beet-sugar factory is dependent on several factors, *viz.* (a) size of grade of filtering material, (b) the amount and nature of the compounds of phosphorus present, (c) the nature of the compounds of nitrogen present.

In order to build up a film able to oxidise a given quantity of carbohydrate at a given rate of flow a filter filled with $\frac{1}{16}$ to $\frac{1}{4}$ in. medium requires a smaller amount of available phosphorus than a filter with a coarse grade of material

such as glass tubes 1.5 cm. long and 1 cm. bore. The primary function of the phosphorus is to supply the nutrient necessary for the growth of enough film to increase the time of passage of the dissolved organic matter through the filter. The results of Exps. 1 and 2 bring out the difference caused by filtering solutions of the same composition through filters filled with different grades of material. Filter B_1 , with a small size of medium, received 0.3 part P_2O_5 per 100,000 and decomposed daily 98 % of a 0.1 % solution of sucrose after the filter had been in operation for one month, while B_2 , which contained a larger grade of medium, but received the same amounts of phosphorus and sugar, oxidised less than 50 % of the sugar after operating for the same period. Filters with too fine a grade of medium suffer from the disadvantage of choking whenever vigorous growth of film occurs. Thus, increasing the supply of phosphorus to 3 parts P_2O_5 per 100,000 in Exps. 1 and 2 led to the choking of the fine medium in filter C_1 , whereas the coarse medium in filter C_2 showed no signs of ponding although it received solution of the same composition as that passed through C_1 . This observation might be of importance in actual practice where the ponding of filters increases operating difficulties and lowers the efficiency of the filter.

When 0.1 % solutions of sucrose containing 0.3 part P_2O_5 per 100,000 were filtered the phosphorus was, with one exception, quantitatively absorbed by the filter. An increase to 3 parts P_2O_5 in the liquids undergoing filtration led to a removal of about 1.5 parts P_2O_5 and the remainder passed out as inorganic phosphate with the effluent. Organic compounds of phosphorus were never detected in the effluents from any sections of the filters, although by the method used [Parker and Fudge, 1927] it was possible to determine as little as 0.002 part P_2O_5 as hexosephosphate per 100,000 of solution. Barritt [1931] treated a 0.2 % solution of sucrose, containing about 23 parts P_2O_5 per 100,000 as mixed potassium phosphates, in a percolating filter and found that the whole of the phosphate was not taken up by the filter. Even when one-sixth of this quantity of phosphate was used, *viz.* about 4.0 parts P_2O_5 per 100,000, he observed that inorganic compounds of phosphorus still appeared in the effluents. The results given in this paper would suggest that the occurrence of phosphate in the effluents from Barritt's filters was the result of supplying the filter with more phosphate than it could remove from solution. No evidence was found to indicate that hexosephosphate appeared in the effluents and became hydrolysed during analysis. Harden [1923] supposes that hexosephosphates are formed within the living cell first by the diffusion of sugar from the substrate through the cell wall, followed by the union of sugar with the phosphate already present in the cell. This compound is then split up into simple products and the phosphate which is regenerated is again able to unite with sugar.

The experiments on the filtration of press water from a beet-sugar factory show that hitherto unsuspected differences may exist as regards the availability of compounds of phosphorus in a percolating filter. In previous work reported by Richards and Cutler [1933] an ample supply of available nitrogen was recognised as essential for the rapid oxidation of dissolved organic impurities. The organic compounds of nitrogen and phosphate in press water probably have varying degrees of availability. Such compounds are removed mechanically by the filter before being assimilated by micro-organisms. The rate of oxidation of the organic matter passing through the filter is limited by the rate at which the nitrogen becomes available and this rate does not seem to be increased by supplying compounds of phosphorus which are readily utilisable by micro-organisms. If, however, nitrogen exists in the filter in an easily assimilable form such as

ammonia, the rate of oxidation of organic matter is considerably increased by the supply of an available form of phosphorus.

Solutions of sugar and ammonium chloride suffered a considerable reduction in p_{H} on filtration, from a figure of 5.4 in the nutrient solution to 3.2 in one effluent, and from 6.0 to 2.8 in another. As previously mentioned, tests for the amount of dissolved organic matter showed that the decrease in p_{H} was too great to be accounted for solely by the production of organic acids from the sugar: negative results were obtained on testing for simple organic acids in the final effluents. It was also observed that the concentration of chloride in the solution undergoing filtration remained practically constant throughout its passage down the filter. Furthermore the p_{H} of the nutrient solution was not reduced appreciably when ammonium bicarbonate was substituted for ammonium chloride. These facts all indicate that the acid production in a percolating filter operated under the conditions described herein is chiefly derived from the mineral acid liberated by the removal of ammonium ion from the chloride or sulphate. Barritt [1931] found that organic acids may be produced in the upper part of a percolating filter if 0.2 % solutions of sugar are filtered: a reduction in p_{H} is observed in that part of the filter but the acid is oxidised as the solutions pass downwards, with corresponding increases in the p_{H} values of the effluents.

SUMMARY.

1. Experiments have been carried out on the biological oxidation in percolating filters of (a) 0.1 % sucrose *plus* different amounts of phosphate as K_2HPO_4 and with NH_4Cl or NH_4HCO_3 as the source of nitrogen, using broken glass as the filtering medium; (b) 0.1 % sucrose *plus* NH_4HCO_3 and different amounts of phosphate, with glass tubes as the filtering medium; (c) diluted press water from a beet-sugar factory; in this case the effect of organic compounds of phosphorus on the oxidation of organic matter was studied in the presence of sources of nitrogen readily available to micro-organisms, and in the presence of compounds of nitrogen available only with difficulty.

2. The amount of phosphate required to ensure rapid oxidation of sugar in a 0.1 % solution is influenced to a large extent by the nature of the filtering medium. To ensure freedom from choking, the medium should be of a large size and of a suitable shape. Under these conditions a 0.1 % solution of sugar requires at first a maximum of about 1.5 parts P_2O_5 and 2.2 parts N per 100,000 for complete oxidation of the sugar; once the film becomes mature 0.3 of P_2O_5 and 1 of N.

3. Excess of phosphate supplied to the filters passes out apparently unchanged. No organic compounds of phosphorus were ever found in the effluents from any of the sections of the different filters.

4. The organic compounds of phosphorus in the press water of a beet-sugar factory are only slowly available to the micro-organisms on a percolating filter as compared with potassium hydrogen phosphate, and this may limit the rate of oxidation of organic matter by a percolating filter. In the absence of a form of nitrogen which is readily assimilated by micro-organisms the full effect of a supply of available phosphorus is not shown.

5. When percolating filters filled with glass medium were supplied with sugar, ammonium chloride and various amounts of K_2HPO_4 the p_{H} values of the solutions before and after filtration were:

- (a) 5.6 and 5.0 respectively, without P_2O_5 ;
- (b) 5.4 and 3.2 respectively, with 0.3 part P_2O_5 per 100,000;
- (c) 5.0 and 2.8 respectively, with 3.0 parts P_2O_5 per 100,000.

The cause of acid production was found to be the removal of NH_3 from NH_4Cl and the liberation of free HCl . Quantitative tests and measurements of the dissolved organic matter present in the effluents showed that this acidity was not due to organic acids.

Acknowledgements are made to Mr E. H. Richards for supervising this work, to Mr D. W. Cutler and staff for permission to refer to unpublished results and to Mr A. S. Roberts for assistance at one stage of the experiments. The investigation was carried out as part of the programme of the Water Pollution Research Board of the Department of Scientific and Industrial Research, and the results are published with the permission of the Department.

REFERENCES.

- Barritt (1931). *Biochem. J.* **25**, 1419.
Harden (1923). Alcoholic fermentation, p. 143. (Longmans, Green and Co., London.)
Jenkins (1933, 1). *Biochem. J.* **27**, 240.
—— (1933, 2). *Biochem. J.* **27**, 245.
—— (1933, 3). *Biochem. J.* **27**, 258.
Ministry of Health (1929). Methods of chemical analysis as applied to sewage and sewage effluents. (H.M. Stationery Office, London.)
Parker and Fudge (1927). *Soil Sci.* **24**, 109.
Richards and Cutler (1933). The purification of waste waters from beet-sugar factories. Water pollution research, technical paper No. 3. (H.M. Stationery Office, London.)
Zinzadé (1932). *Z. Pflanz. Dung. Boden.* **23 A**, 447.

THE PREFERENTIAL UTILIZATION OF DIFFERENT FORMS OF INORGANIC NITROGEN IN THE DECOMPOSITION OF PLANT MATERIALS

E. H. RICHARDS AND J. G. SHRIKHANDE

Rothamsted Experimental Station, England

Received for publication February 21, 1934

The experiments described in this paper were carried out with the object of discovering whether the organisms concerned in the decomposition of cellulosic matter exercise any preference for ammoniacal or nitric nitrogen when both forms are available to them in equal concentration.

It is now well established that practically any form of nitrogen will supply the needs of the fungi and bacteria which break down polysaccharide carbohydrates to humus and, in so doing, narrow the wide carbon-nitrogen ratio of the raw material.

Although almost any kind of nitrogen can be used, there are very marked differences in the effects brought about by the process of decomposition. For example, the maximum temperature reached, the color and physical condition of the end product, and the loss of elementary nitrogen sustained are all influenced by the form in which the nitrogen is supplied.

When the carbohydrate is decomposed in the soil it may easily happen that, in addition to the soil nitrate, much nitrogen is also present as ammonia. Such conditions are found when fresh unrotted farmyard manure is ploughed under, or when raw straw is returned to the soil together with a dressing of sulfate of ammonia.

The reactions of soil nitrogen to applications of strawy manure and to raw straw have been studied by many workers, and an extensive literature is devoted to this question. Only a few of these papers will be referred to here.

Niklewski (8) studied the effect of straw on the utilization of nitrogen in various compounds, including ammonium sulfate and sodium nitrate, by an oat crop grown in pots. Only one form of nitrogen was used in each pot, and attention was directed chiefly to effects of concentration and distribution in the soil. This worker, however, noted that straw had an unfavorable influence on the utilization of ammonium sulfate in lower concentrations but that the reverse was the case with similar concentrations of sodium nitrate. In other words, Niklewski found that nitrogen in the form of ammonium sulfate was rapidly immobilized by combination with straw so that the oat crop suffered, whereas sodium nitrate was, apparently, more completely assimilated by the crop in the presence of straw than in its absence. This last observation is rather surprising, but the results are in the same direction as those described in this paper.

Among others, Scott (10), Collison and Conn (2), Murray (6), and Gilbert and Pember (3) have all noted that the application of straw to soil results in a reduction of nitrate with consequent depression of crop yield. This effect could be overcome by addition of sufficient nitrogenous manure. The question of preference for any particular form of nitrogen is not discussed.

EXPERIMENTAL

In the first instance the reactions of ammonia and nitrate with straw were tested alone, unmixed with soil. It is hoped to continue the experiments with nitrogen and straw in both light and heavy soils.

Experiment 1

Air-dried chaffed wheat straw in lots of 20 gm. was well moistened by spraying. Sufficient solution of ammonium nitrate was added to each bottle to supply 1.0 per cent of nitrogen to the straw, half as ammonia and half as nitrate. The additional inorganic nitrogen immobilized as organic nitrogen by 100 gm. of any material in process of decomposition has been termed (9) the "nitrogen factor." As the nitrogen factor of normal wheat straw does not usually exceed 0.8 per cent there would then be a small excess of available nitrogen over and above that immobilized after rotting was complete.

Each bottle contained originally 18.5 gm. of dry matter as straw and 70 cc. water. The bottles were plugged with cotton wool and incubated at 35°C. Since the straw had 0.315 per cent nitrogen in dry matter, the bottles each contained 0.0582 gm. nitrogen in straw and 0.200 gm. nitrogen as ammonium nitrate, or 0.2582 gm. in all.

A bottle was removed at intervals over a period of 160 days for the determination of dry matter and ammoniacal, nitric, and total nitrogen. Ammonia was distilled from a sample made alkaline with magnesia, and nitrate was estimated in the residue by reduction with Devarda's alloy. These methods are known to give high values for both ammonia and nitrate when used on samples containing easily decomposed organic nitrogen. After the thirtieth day, for example, no blue coloration could be found with diphenyl benzidine (5), although the Devarda figure showed 0.04 per cent of nitric nitrogen in the wet sample. The color test is sensitive to one part in a million and it is certain that no nitrate was really present after the thirtieth day. The excess of apparent ammoniacal nitrogen comes, of course, in both cases, from the organic nitrogen and is not originally present as either ammonia or nitrate. More refined methods were used in the second series of experiments with the result that the values for both ammoniacal and nitric nitrogen after 28 days' incubation fell to about one-tenth of those found in the preliminary series.

The figures given in table 1 show clearly that from the start both forms of nitrogen are utilized by the organisms. During the first 15 days, however, when the fungal development is most active, there is a small but definite preference for ammonia over nitrate. On the eighth day, for example, 62 per cent

of the ammoniacal nitrogen but only 33 per cent of the original nitric nitrogen had been removed. In the later stages there is an approximately equal utilization of both forms, but it is very important to remember that the gradual reduction of nitric nitrogen is due to loss as elementary nitrogen as well as to assimilation, whereas the ammoniacal nitrogen is all immobilized if not supplied in excess of the nitrogen factor of the cellulosic base. This difference is more clearly shown in the second series of experiments.

Experiment 2

In this experiment four different sets of bottles, each containing 20 gm. of air-dry oat straw, were incubated for 56 days. Nitrogen was supplied as (a) ammonium carbonate, (b) ammonium nitrate, (c) sodium nitrate and (d) a mix-

TABLE 1
Wheat straw rotted with ammonium nitrate at 35°C.
Calculated on 100 gm. of original dry straw

DAYS	LOSS OF DRY MATTER	NITROGEN PRESENT AS			NITROGEN FACTOR
		NH ₃	NO ₃	Total	
	gm.	gm.	gm.	gm.	per cent
0	0.54	0.54	1.40
1	Nil	0.42	0.55	1.37	0.09
3	3.9	0.44	0.50	1.43	0.18
8	20.4	0.21	0.36	1.26	0.40
15	25.6	0.28	0.31	1.32	0.43
22	29.1	0.23	0.28	1.32	0.52
30	36.2	0.19	1.09	0.56
36	36.0	0.19	0.21*	1.28	0.59
136	56.2	0.13	0.22*	1.14	0.74
160	48.8	0.11	1.17	0.78

* These figures are certainly too high for the reason explained in the text.

ture of (a) and (c). As 0.20 gm. of nitrogen was added to each bottle there was present at the start an excess over the amount expected to be immobilized in rotting. After the 20 bottles of this series had all been analyzed, a final set of five bottles of oat straw was incubated to which 0.32 gm. nitrogen, i.e., more than double the nitrogen factor, was added as ammonium nitrate. The object of this experiment was to determine whether a supply of nitrogen in the form of either ammonia or nitrate sufficient to complete the reaction without calling on the other would influence the result.

Ammoniacal and nitric nitrogen were estimated as follows:

Ammonia. The method was devised by Nichols and Foote (7) for the estimation of free ammonia in sewage and trade wastes. A phosphate buffer solution was made up by dissolving 14.3 gm. KH₂PO₄ and 90.15 gm. of K₂HPO₄·3H₂O in distilled water made up to 1 liter. About 10 gm. of the wet manure were

distilled from 300 cc. of water with 25 cc. of the phosphate buffer solution. In this way the separation of the free ammonia from the organic nitrogen was conducted at a pH of about 7.4, which was maintained constant by the phosphate buffer.

Nitrate. This was estimated by Bengtsson's method (1). Ten grams of the wet manure were extracted with 300 cc. of distilled water in fractions of 50 cc. Each fraction was allowed 10 minutes' extraction with occasional stirring. The supernatant solution was decanted through a cotton wool plug filter. This process was repeated until the extract no longer gave a blue coloration with diphenyl benzenedene. The suspended organic matter and colloids were precipitated with a few drops of H_2SO_4 , and the solution was warmed. The settled matter was filtered off on a Buchner funnel under suction. The filtrate was made alkaline to litmus with NaOH and evaporated down to about 30 cc. After being made up to 200 cc., including 25 cc. of 10 per cent NaOH, the filtrate was again evaporated to 30 cc. By this time any ammonia nitrogen originally present as such and any produced from organic nitrogen in the extract had been volatilized. Usually one evaporation was sufficient. The residue was finally distilled from about 300 cc. of water and Devarda's alloy and the ammonia absorbed in standard acid as usual.

The effect of the improved methods of estimating ammonia and nitrate is clearly seen in table 2. After the fourteenth day of the experiment the amount of ammoniacal nitrogen is about one-tenth of that found in the first experiment (table 1) after an equal period of rotting. The nitric nitrogen is also reduced to a similar extent. Comparison is possible only with the ammonium nitrate series in experiment 2, but the values for inorganic nitrogen are consistently low in the later stages of all the series in which other nitrogen compounds were under test.

The preference of the organisms for ammoniacal nitrogen in the first 14 days shown by the preliminary experiment is confirmed in the two series of experiment 2 in which the nitrogen was supplied as ammonium nitrate and as a mixture of ammonium carbonate and sodium nitrate. Further, if the figures for series (a) ammonium carbonate, are compared with series (c) sodium nitrate, it will be noticed that on the seventh day the ammoniacal nitrogen has been reduced by 96 per cent but the nitric nitrogen by only 59 per cent. By the fourteenth day the maximum amount of nitrogen had been converted to protein in all three series where nitrate was originally added, after that the nitrogen factor declines and the residual inorganic nitrogen is usually less than 0.10 per cent of the dry matter in the sample. On the other hand when the nitrogen is supplied entirely as ammonia the nitrogen factor remains at a maximum up to the fifty-sixth day, and the percentage of nitrogen in the final product is higher than in any other samples of this experiment. At this stage the ammonification of protein has begun, so that rather more ammonia is found on the fifty-sixth than on the twenty-eighth day, irrespective of whether the nitrogen was originally supplied as ammonia or nitrate.

TABLE 2

Oat straw rotted with different nitrogen compounds at 35°C.

Calculated on 100 gm. of original dry straw

DAYS	DRY MATTER	NITROGEN PRESENT AS			LOSS OF N	NITROGEN FACTOR
		NH ₃	NO ₂	Total		
	gm.	gm.	gm.	gm.	per cent	per cent
<i>(a) Ammonium carbonate</i>						
0	100.0	1.15	1.64
3	100.0	0.84	1.35	17.8
7	94.2	0.05	1.27	22.7	0.28
14	70.7	0.04	1.35	18.0	0.81
28	60.1	0.03	1.25	23.9	0.72
56	43.6	0.06	1.40	14.8	0.85
<i>(b) Ammonium nitrate</i>						
0	100.0	0.57	0.57	1.64
3	100.0	0.32	0.49	1.27	22.5
7	89.1	0.05	0.15	1.21	26.9	0.51
14	65.1	0.03	0.04	1.34	18.6	0.78
28	57.0	0.02	0.01	1.11	32.2	0.59
56	40.4	0.05	0.03	1.15	29.9	0.58
<i>(c) Sodium nitrate</i>						
0	100.0	1.15	1.64
3	100.0	0.03	1.01	1.28	22.2
7	92.2	0.03	0.47	1.11	32.2	0.11
14	76.3	0.02	0.15	1.14	30.5	0.48
28	62.6	0.02	0.02	1.01	38.4	0.47
56	47.4	0.04	0.02	0.92	44.4	0.36
<i>(d) Ammonium carbonate and sodium nitrate</i>						
0	100.0	0.57	0.57	1.64
3	100.0	0.36	0.48	1.31	19.7
7	86.3	0.04	0.12	1.09	33.6	0.44
14	73.9	0.03	0.03	1.31	20.2	0.77
28	62.0	0.02	0.01	1.17	28.7	0.64
56	43.6	0.04	0.02	1.08	34.6	0.52
<i>(e) Ammonium nitrate (higher concentration)</i>						
0	100.0	0.93	0.93	2.27
3	96.1	0.46	0.44	1.32	41.7
7	92.7	0.30	0.28	1.32	41.7	0.29
14	73.2	0.06	0.03	1.39	38.8	0.86
28	61.7	0.05	0.04	1.47	35.8	0.94

As the loss of nitrogen during the rotting of the various samples of straw was often considerable and this question is of practical importance, the conditions which gave rise to the greatest loss are worth noting. Throughout the whole experiment, with the exception of the final series with a higher concentration of ammonium nitrate, nitrogen was added in excess of the normal nitrogen factor of the oat straw (0.85) to the extent of 25 per cent. This amount is therefore the greatest loss to be expected if no inorganic nitrogen remained unassimilated. Allowing for the ammonia not volatilized, this result is substantially found in series 2 (a) in which ammonium carbonate was used. In the other series, all containing nitrate, there is a greater loss of nitrogen which is at a maximum of 44 per cent in series 2 (c) (sodium nitrate). Further, when nitrate is present the nitrogen factor is always lower than when the nitrogen is supplied entirely as ammonia. There seems to be some cause which inhibits the growth of those organisms, probably fungi, responsible for the high proportion of protein found when ammonia is the only source of nitrogen. This effect was most marked in series 2 (c) (sodium nitrate). There the nitrogen factor is only a little more than one-half the normal value. The trouble is not due to shortage of mineral nitrogen, for plenty of unused nitrate is present on the fourteenth day when the nitrogen assimilated was at a maximum. Possibly the alkalinity of the rots with NaNO_3 (pH 9–10) may be partly responsible, but the effect is noticeable in series 2 (b) (ammonium nitrate), where the reaction was approximately neutral. Nitrate does not check the oxidation of the carbohydrates—only the development of protein. Indeed the greatest loss of dry matter in the whole series of experiments, almost 60 per cent, was found in series 2 (b) (ammonium nitrate).

Very soon after the first trials of artificial farmyard manure had been made (4) it was recognized that from the economic point of view nitrate was one of the least desirable forms of nitrogen for that purpose. Apart from its high cost per unit of nitrogen the possibility of denitrification must always be present. The experiments described in this paper show clearly how large these losses of nitrogen may be even in bottles where no leaching can occur. Manures made with nitrate nitrogen, in whole or in part, are inferior to those made with ammonium carbonate, as judged by the total nitrogen in the final dry matter and by the nitrogen immobilized per 100 gm. of original straw (nitrogen factor). In the case of sodium nitrate used alone the comparison is particularly unfavorable to nitrate.

Besides richness in protein the yield of manure is also important, and in this respect there is little difference whatever form of nitrogen is used. The loss of dry matter after 28 days is about 40 per cent in all cases. At this stage, equivalent to about four months in a manure heap under average conditions, the products are generally at their best for application to the soil.

When strawy unrotted farmyard manure is applied to a soil containing a reserve of nitrate the organisms have a choice of using either the ammoniacal

nitrogen in the urine-saturated straw or calling on the soil nitrate. These experiments suggest that the ammonia will at first be utilized but that some nitrate will also be built up into protein in spite of the fact that there is more than sufficient ammoniacal nitrogen to satisfy all requirements. At the same time some of the nitrate nitrogen will probably be lost as elementary nitrogen.

A similar series of changes will no doubt occur when raw straw is ploughed into the soil along with sufficient available nitrogen to avoid the depression of crop yield which otherwise follows the immobilization of the soil nitrates. The straw will draw mainly on the ammonia, but some nitrate will also be taken with the usual associated loss of elementary nitrogen.

It is hoped to make some experiments with straw and ammonium-nitrate mixtures in both light and heavy soils to test the validity of the hypotheses submitted.

SUMMARY

When straw is in contact with both ammoniacal and nitric nitrogen in equal initial concentration under conditions favorable for decomposition there is a definite preference by the organisms concerned in the earlier stages of breakdown for ammonia rather than for nitrate.

After 14 days at 35°C. unassimilated inorganic nitrogen is about equally divided between ammonia and nitrate. There is then no apparent preference for either form.

When nitrogen is supplied wholly or partially as nitrate the nitrogen factor calculated after rotting is complete is always lower than when ammonia is used.

The loss of nitrogen is always greatest when nitrate is present. Sodium nitrate and ammonium nitrate lost 30 and 15 per cent more nitrogen, respectively, than ammonium carbonate in equal original concentration.

As a result of this loss of, presumably, elementary nitrogen from nitrate, the relative assimilation of ammonia may be greater than the figures indicate, since the drop in nitrate includes both the nitrogen lost as well as that assimilated.

Some practical applications of these results are discussed.

REFERENCES

- (1) BENGTSSON, N. 1932 Determination of nitrates in soil and manure. *Meddel. 415 Centralanst. Försökv. Jordbruksområdet. Bact. Avdelningen* 58: 3-41.
- (2) COLLINSON, R. C., AND CONN, H. J. 1925 Effect of straw on plant growth. N. Y. (Geneva) Agr. Exp. Sta. Tech. Bul. 114.
- (3) GILBERT, B. E., AND PEMBER, F. R. 1933 Influence of rye and oat straws upon the growth and yield of certain vegetables. *Soil Sci.* 35: 115-122.
- (4) HUTCHINSON, H. B., AND RICHARDS, E. H. 1921 Artificial farmyard manure. *Jour. Min. Agr.* 28: 398-411.
- (5) LETTS, E. A., AND REA, F. W. 1914 An extremely delicate colorimetric method for detecting and estimating nitrates and nitrites. *Jour. Chem. Soc.* 105: 1157-1161.
- (6) MURRAY, T. J. 1921 Effect of straw on biological soil processes. *Soil Sci.* 12: 233-259.

- (7) NICHOLS, M. S., AND FOOTE, M. E. 1931 Distillation of free ammonia nitrogen from buffered solutions. *Indus. and Engin. Chem. Anal. Ed.* 3: 311-313.
- (8) NIKLEWSKI, B. 1915 Influence of the distribution of nitrogenous fertilizers and straw in soil on plant production. *Zischr. Landw. Versuchsw. Österr.* 18: 674-690.
- (9) RICHARDS, E. H., AND NORMAN, A. G. 1931 Biological decomposition of plant materials: V. *Biochem. Jour.* 25: 1769-1778.
- (10) SCOTT, H. 1921 Influence of wheat straw on the accumulation of nitrates in the soil. *Jour. Amer. Soc. Agron.* 13: 233-258.

A STUDY OF THE COMPOSITION AND UTILISATION OF ALBERTA PEATS

By J. D. NEWTON, PH.D.

(From the General Microbiology Department, Rothamsted
Experimental Station, Harpenden, Herts.)

NUMEROUS peat or "muskeg" areas are to be found in the wooded soil belt of Alberta, Saskatchewan and Manitoba. The peat areas occur mainly in the northern parts of these provinces. In Manitoba, however, east of the main cultivated wheat area, peat occurs as far south as the United States border; and in Alberta peat areas are found west of the main cultivated area as far south as a point about 200 miles north of the United States boundary. With the information available it is not possible to estimate the extent of the peat areas at all accurately, but it has been estimated that there are 37,000 square miles of peat bogs in Canada and that a large proportion of this land lies in Alberta, Saskatchewan, Manitoba and the adjacent territories(4).

Peat areas consist mainly of organic matter, whereas ordinary soils consist mainly of mineral matter. The peats of Western Canada are usually underlaid by clay or other soil material fine enough to hold the moisture in basins or flats. These basins permitted and encouraged the growth of sphagnum moss and other moisture-loving plants. The accumulation of these plants under water-logged conditions resulted in the formation of peat bogs. The "muskegs" are generally covered by a shrubby growth of Labrador tea (*Ledum groenlandicum*), and frequently there are spruce and tamarack trees growing in the peat areas. Studies of the vegetation of Alberta peat areas have been made by members of the Botany Department of the University of Alberta(7).

Very little experimental work with peats has as yet been done in Western Canada, and we are quite uncertain as to the best methods of reclaiming them or bringing them under cultivation. However, some areas, particularly of shallow peat land, have already been brought under cultivation.

Large areas of peat land have been reclaimed in Denmark, Germany, Sweden and other countries of Europe, and much experimental work has been done at the Bremen peat experimental station, and at other

252 *Study of Composition and Utilisation of Alberta Peats*

experimental stations in Europe. In England many thousands of acres of "fen" land have been reclaimed. Pioneer work on reclamation of peat land was done in Scotland, and recently experimental work on the reclamation, cultivation and composition of certain Scottish peat lands has been undertaken by the Macaulay Institute for Soil Research (9). Large areas of peat are found in various parts of the United States, and considerable reclamation work has been done in Minnesota and other states. The American Peat Society was formed in 1907.

The Alberta peats studied would be classified as "highmoor" or "moss" peats. Chemical studies of "highmoor" peat profiles of the Eastern United States, including determinations of the organic constituents, have been made by Waksman and Stevens (12).

The following recent work, also, has a bearing upon the experiments reported in the paper. Du Toit and Page (3) have studied the carbon and nitrogen cycles in soil, and the formation of natural humic matter; and Jensen (6) has studied the microbiology and chemistry of farmyard manure decomposition in soil. In both cases some work on decomposition of filter paper cellulose was done. In studies of the biological decomposition of plant materials, Norman (8) found that in general all substances in straw but lignin were attacked by different fungi to a degree relatively proportional to the apparent total loss of organic matter.

The peats of the Canadian prairie provinces generally possess a rather thick surface layer of light-coloured and but slightly decomposed peat. Therefore it will probably require considerable time after drainage to decompose them sufficiently to bring them into a satisfactory condition for the growth of good crops. Application of nitrogen, phosphate and potash fertilisers and farmyard manure will probably prove beneficial. Peats vary in reaction but many of them are very acid, and such peats should respond to treatments with lime, or marl, from local deposits. Their fertiliser requirements may best be determined by analyses, laboratory and greenhouse culture experiments, and field trials.

The underlying layers of peat are darker in colour, decomposed to a greater degree, and probably closer to the productive condition. Therefore, we are mainly concerned with the surface layer which must be decomposed to a considerable degree in order to produce a productive soil.

Preliminary determinations made by the writer at Rothamsted showed that this surface layer was rich in cellulose, and it is believed that this excess of cellulose must be decomposed in order to make the peat

productive. While waiting for samples of peat large enough for satisfactory fermentation experiments to come from Canada an experiment on decomposition of relatively pure cellulose was started and carried on. The object was to determine whether the elements commonly applied as fertilisers in field practice (nitrogen, phosphorus and potash) would bring about cellulose decomposition when applied to a comparatively pure form of cellulose. The cellulose used was ground filter paper which contained very little ash (Whatman No. 1—a 24.0 cm. dia. sheet contains 0.0136 gm. ash).

CELLULOSE FERMENTATION EXPERIMENT.

The treatments were as follows: (1) control, moisture only; (2) nitrogen in the form of ammonium carbonate, $(\text{NH}_4)_2\text{CO}_3 \cdot \text{H}_2\text{O}$, at the rate of 1.0 g. nitrogen per 100 g. water-free cellulose; (3) nitrogen and phosphorus in the forms of ammonium phosphate $(\text{NH}_4)_2\text{HPO}_4$, and ammonium carbonate, at the rate of 1 gm. nitrogen and 0.4 gm. phosphorus per 100 gm. water-free cellulose; (4) nitrogen, phosphorus and potassium in the forms of ammonium carbonate, and potassium phosphate, K_2HPO_4 , at the rate of 1.0 g. nitrogen, 0.4 g. phosphorus, and 1.0 gm. potassium per 100 gm. water-free cellulose; (5) nitrogen, phosphorus, potassium, magnesium, sulphur, calcium, and iron in the forms of ammonium carbonate, potassium phosphate, magnesium sulphate, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, calcium carbonate, CaCO_3 , and ferrous sulphate, FeSO_4 , at the rate of 1.0 gm. nitrogen, 0.4 gm. phosphorus, 1.0 gm. potassium, 0.25 gm. magnesium, 0.25 gm. sulphur, 1.0 gm. calcium, and a trace of iron per 100 gm. water-free cellulose.

Conical flasks of 750 c.c. capacity were used, and there were five treatments in quadruplicate. Fifty grams of water-free cellulose were placed in each, and enough water was added to bring the moisture content up to 80 per cent. of the moisture-holding capacity of the cellulose. No more moisture was added during the incubation period, as the cultures lost moisture very slowly. Aeration was provided by placing cotton-wool in the holes cut through the rubber stoppers. Each culture was then inoculated with a small quantity of water extract from a fertile soil to make sure that sufficient micro-organisms were present. The cultures were incubated in a warm room at a temperature of about 30° C.

At the end of the incubation period the pH values of the different cultures were approximately as follows: first pH 7.0–7.5; second pH 9.5; third pH 8.0; fourth pH 8.0; fifth pH 7.0–7.5. These reactions are satisfactory for microbiological activity.

254 *Study of Composition and Utilisation of Alberta Peats*

After 45 days' incubation and again after 107 days' incubation duplicate flasks of each treatment were removed and the residues placed on a Buchner funnel covered with poplin cloth, and washed with hot water. The remaining insoluble residues were dried and weighed.

The experimental results given in Table I show very clearly that under the conditions of this experiment (and probably under any condition) the three elements commonly applied in the form of mineral fertilisers in farm practice will not produce rapid decomposition of cellulose. Even after 107 days these elements had produced very little decomposition, whereas the decomposition was rapid in the cultures to which the other "essential" elements were added.

Table I.
*Weights of oven-dried solid residues from 50 gm.
cellulose after fermentation.*

Treatment	After 45 days' incubation gm.	After 107 days' incubation gm.
Control	50.0	49.5
Control	50.4	49.5
N	49.5	49.1
N	49.9	49.5
N + P	49.0	44.0
N + P	48.2	43.9
N + P + K	47.4	46.8
N + P + K	46.7	45.5
N + P + K + Mg + S + Ca + Fe	34.6	19.3
N + P + K + Mg + S + Ca + Fe	33.3	18.5

Repeated attempts were made to determine the numbers of cellulose-decomposing bacteria in these cultures by diluting and plating on cellulose-mineral-agar media, but it was doubtful if the colonies developing on the plates were cellulose decomposers, and satisfactory counts could not be made. It is suggested that the rapid decomposition of cellulose in the cultures to which all "essential" elements were added was brought about mainly by fungi.

At the end of the longer incubation period (after 107 days) the cultures were plated out on a medium which would encourage the growth of a wide variety of ammonifying bacteria. The medium contained 15 gm. agar, 10 gm. peptone, 3 gm. "Lemco" meat extract, and 5 gm. sodium chloride per litre of distilled water. Whether any of the bacteria developing colonies on this medium would be capable of decomposing cellulose is, of course, uncertain. No doubt many of them would be concerned with secondary stages of decomposition.

The relative numbers of bacteria from the different cultures are shown in Table II. Each number was obtained by taking the average of five replicate plates and multiplying by the dilution used. There is no close correlation between the counts shown in Table II and the rates of cellulose decomposition shown in Table I. For example, the addition of nitrogen alone greatly increased the bacterial numbers, but there was no significant destruction or discoloration of the cellulose in this culture. However, it will be observed that the numbers increased with each additional "essential" element, and that the culture to which all "essential" elements were added, and which decomposed rapidly, contained the largest number of bacteria.

Table II.

Plate counts of bacteria in the different cultures at the end of the 107-day incubation period.

Culture	No. per gm. of original cellulose
Control	730,000
N	10,160,000
N + P	87,000,000
N + P + K	192,000,000
N + P + K + Mg + S + Ca + Fe	630,000,000

PEAT EXPERIMENTS.

Different horizons or layers of the Alberta peats studied differ greatly in colour or stage of decomposition and in reaction or lime requirement, as is shown below:

Carnwood peat. Surface layer, light in colour, and very acid in reaction (about pH 4.0).

Winterburn peat. Surface layer, light in colour and nearly neutral in reaction (pH 7.0-7.5). This layer is about 2 ft. thick. Subsurface layer, dark in colour and nearly neutral in reaction (pH 7.0-7.5). This layer is about 2 ft. thick. Deep layer, very dark in colour and nearly neutral in reaction (about pH 6.5). This layer lies just above the clay underlying this peat area.

Spruce Grove peat. Surface layer, very light in colour and very acid in reaction (about pH 4.0). Subsurface layer, light in colour, and nearly neutral in reaction (pH 7.0-7.5).

Stonyplain peat. Surface layer, light in colour, and nearly neutral in reaction (pH 7.0-7.5). This layer is about 2 ft. thick. Subsurface layer, darker than surface in colour and somewhat acid in reaction (about pH 5.5). This sample was taken at a depth of 4 ft.

256 *Study of Composition and Utilisation of Alberta Peats*

Ash. Widely different amounts of ash were obtained on ashing these various samples of Alberta peat, as shown in Table III. The surface samples usually contain less ash than the deeper samples because the surface samples are less decomposed. The Stonyplain peat is exceptional in this respect, but this may be due to marl washed over the surface, because it was observed that the surface sample was neutral in reaction and the deep sample somewhat acid. The very high ash content of the deepest sample of Winterburn peat is probably caused by an admixture of underlying clay with the peat.

Table III.

Composition of peat samples (water-free basis).

Peat sample	Total ash %	Cellu- lose (minus ash) %	Lignin (minus ash) %	Cellu- lose + lignin + ash %	Ether extract %	Nitro- gen N %	Phos- phorus P ₂ O ₅ %	Cal- cium CaO %	Reaction pH
Carnwood, 1st depth	4.1	26.0	35.6	65.7	0.7	1.07	0.13	0.90	4.0
Winterburn, 1st depth	7.6	33.5	38.6	79.7	1.1	1.02	0.11	3.43	7.0-7.5
Winterburn, 2nd depth	10.4	5.1	44.1	59.6	0.6	2.51	0.18	3.29	7.0-7.5
Winterburn, 3rd depth	37.5	0.0	42.1	79.6	0.6	1.75	0.27	1.07	6.5
Spruce Grove, 1st depth	14.0	46.9	20.1	81.0	1.1	0.46	0.10	1.19	4.0
Spruce Grove, 2nd depth	20.1	30.2	25.9	76.2	1.0	0.79	0.13	4.61	7.0-7.5
Stonyplain, 1st depth	21.1	21.1	24.6	66.8	0.5	0.89	0.13	4.75	7.0-7.5
Stonyplain, 2nd depth	8.1	11.8	48.9	68.8	0.9	1.74	0.21	3.47	5.5
Vimy, 1st depth	—	54.5*	—	—	—	—	—	—	—
Vimy, 2nd depth	—	11.0*	—	—	—	—	—	—	—

* Including cellulose ash.

Nitrogen. The nitrogen content of these samples of Alberta peat also varies rather widely (Table III). The subsurface layer usually contains about twice as much nitrogen as the surface layer. The explanation for this is that the subsurface layer has been decomposed to a greater degree than the surface layer, and carbon is lost more rapidly than nitrogen on decomposition of raw peat. It is interesting to observe that in ordinary mineral soils the opposite condition holds true: the surface layer nearly always contains more nitrogen than the subsurface because the subsurface contains less organic matter. The nitrogen, calcium and total carbon content of a peat profile obtained near Winterburn, Alberta, was reported by Bedford (2) and in this case, also, the deep sample contained more nitrogen than the surface sample.

It is probable that peats rich in nitrogen will be more quickly reclaimed, or brought into a condition satisfactory for the growth of good crops, than peats poor in nitrogen, other conditions being equal, since a higher nitrogen content usually means a greater degree of decomposition.

Phosphorus. The phosphorus content of the different samples of peat does not vary as much as the nitrogen content (Table III). The sub-surface layer in each case contains more phosphorus than the surface, probably because the subsurface is more decomposed.

Peats reclaimed for crop production are frequently deficient in available phosphorus. The availability of the phosphorus in these peat samples has not, as yet, been determined.

Calcium. The calcium content of these samples of Alberta peat varies from less than 1 per cent. to nearly 5 per cent. (Table III).

According to Alway and Nygard(1) peats especially in need of lime narrow down to those with pH values of 4.5-3.5, but, according to these investigators, pH values do not seem to bring out differences in lime requirements as well as percentages of calcium. Peats which contain 2 per cent. or more of calcium oxide are not likely to require lime for satisfactory crop production.

Judging by the calcium oxide percentages and the pH values the Carnwood and Spruce Grove peats require liming, whereas the Winterburn and Stonyplain peats do not require liming for satisfactory crop production.

Organic constituents. Cellulose and lignin form the major part of the organic matter added to soil, and there is usually a good deal more cellulose than lignin in the plant residues added. Cellulose is a hexosan which on hydrolysis yields glucose. Lignin is built up from complex benzene ring groupings, but its chemical structure is not definitely known. Lignin is chemically very resistant, and, by treating organic matter with strong acids other substances are destroyed.

Cellulose was determined by the Jenkins method(5) a convenient modification of the standard one of Cross and Bevan. The Jenkins method, as used for the determination of cellulose in straws, calls for boiling and extracting the straw twice with a solution of approximately 1 per cent. sodium hydroxide, and twice with hydrochloric acid; but it was decided that four successive extractions with alkali and acid were required for the peat samples. The method also calls for chlorinating the straw twice with a definite solution of sodium hypochlorite, each time for a period of 20 min., but it was found necessary to chlorinate the peat samples for several hours instead of 20 min. each time (once overnight as a rule). Possibly fewer extractions with alkali and less chlorination would have been better for certain samples, as some loss of cellulose may have occurred, but it was decided that all samples should be treated alike for purposes of comparison.

258 *Study of Composition and Utilisation of Alberta Peats*

Lignin was determined by the method of Ost and Wilkening as, modified by Schwalbe (10). Two grams of the dried material are treated with 60 c.c. of 72 per cent. sulphuric acid and 15 c.c. of 18 per cent. hydrochloric acid and kept in an ice-box for 24 hours. At the end of this period about 500 c.c. distilled water are added, and the whole is boiled for half an hour to complete the hydrolysis. The product is filtered through paper on a gooch crucible, washed, dried, weighed and ashed. The weight of lignin is given by subtracting the weight of ash.

The cellulose content of the peats varies from none to about 47 per cent. of ash-free cellulose (Table III). In the four cases in which comparisons of surface and lower layers of peat can be made, the surface layer contains more cellulose than the lower horizons; and, in the case of the Winterburn peat, in which three depths can be compared, the second depth contains much less than the surface layer, and the dark coloured, deep third layer has lost all of its cellulose. It will be observed that the sample which has the highest percentage of ash-free cellulose has the lowest percentage of lignin. It will also be observed that the three samples which contain more than 40 per cent. of lignin are the three lowest in cellulose. A decrease in cellulose content is therefore usually accompanied by an increase in per cent. lignin. This is to be expected if we assume that when plant residues decompose, the cellulose decomposes more readily than the lignin, the latter being relatively resistant to decomposition.

The cellulose determined might be defined as Cross and Bevan cellulose, since the Jenkins method gives results which are practically identical with those obtained by the Cross and Bevan method. Some hemicellulose may be retained, but, as previously suggested, some loss of cellulose may have occurred also. The percentages of cellulose found in the surface horizons of the Spruce Grove and Vimy samples are very high.

It will be observed that cellulose lignin, and ash together make up about two-thirds or more of the weight of the peat. In the one case in which the fraction is smaller (the Winterburn second depth sample), the percentage of nitrogen or nitrogenous organic matter, is exceptionally high.

The remaining fraction of the peat would be made up of a variety of substances. Nitrogenous organic matter would account for about 3 to 16 per cent. of the total dry weight of the peat, if we assume that the nitrogenous organic matter contains about 16 per cent. of nitrogen. Hemicelluloses were not determined and might account for a considerable percentage of the remaining peat.

The samples were extracted for 5 hours with petroleum ether (boiling-point not higher than 40° C.), in a Soxhlet apparatus, and the extract was evaporated, dried and weighed. However, the material soluble in petroleum ether accounts for only 1 per cent. or less of the total peat (Table III).

EXPERIMENT TO STUDY FERTILISER REQUIREMENTS.

A laboratory peat fermentation experiment was started with the object of studying, in a general way, the fertiliser requirements of a sample of Alberta peat. The surface sample of Carnwood acid peat was used.

Cultures (1) without fertilisers, (2) with nitrogen alone, (3) with phosphorus alone, (4) with nitrogen and phosphorus together, and (5) with nitrogen, phosphorus and potassium together, all with and without lime, were prepared. Each treatment was in duplicate. The fertiliser salts were added in relatively small quantities, comparable to quantities added in field fertiliser practice. Nitrogen was given in the form of ammonium sulphate ($(\text{NH}_4)_2\text{SO}_4$), phosphorus in the form of calcium acid phosphate ($\text{CaH}_4(\text{PO}_4)_2 \cdot \text{H}_2\text{O}$), and potassium in the form of potassium sulphate (K_2SO_4), at the rate of 0.1 gm. of each of these three elements per 100 gm. of peat. Lime was added at the rate of 2.0 gm. calcium carbonate per 100 gm. peat.

The peat was ground coarsely and placed in 750 c.c. culture flasks at the rate of 50 gm. water-free peat per flask. The optimum quantity of moisture and the desired salts were added and the cultures were fermented in a warm room at a temperature of approximately 30° C. Aeration was provided by placing cotton-wool in the holes cut through the rubber stoppers, but the cultures were not stirred during the period of incubation.

After incubating the cultures for a period of 94 days, a portion of the peat in each flask was placed in separate pots. The remaining portion of each cultures was left to be plated out for bacterial counts at a later date. Oats were planted in each pot and grown in a greenhouse for a period of 24 days. The young oat plants were then harvested, dried, and weighed. However, the differences in weight were not considered to be significant.

After 110 days' incubation the peat cultures were plated out on a medium which would encourage the growth of ammonifying bacteria. The dilution used was 1 to 100,000, but the numbers of bacteria present proved to be small, averaging less than four colonies per plate. The

260 *Study of Composition and Utilisation of Alberta Peats*

numbers were too small for satisfactory counts, and they indicate that there was little bacterial activity in the cultures at the end of the incubation period. The number of fungal colonies developing on the plates was greater than the number of bacterial colonies, and it seems probable that fungal activity was greater than bacterial activity.

The growth of the oat seedlings and the bacterial plate counts show that the fertility of this surface peat was not greatly affected by the small additions of fertiliser salts. Possibly a greater effect upon plant growth might have been produced if the seed had been planted at the time of adding the fertiliser salts. This is a question which should be investigated. The experiment indicated that surface peats of this nature with a high percentage of cellulose are not easily decomposed, and suggests that the process of reclamation for satisfactory crop production will require a period of years.

EXPERIMENT TO STUDY THE POSSIBILITY OF BRINGING ABOUT RAPID DECOMPOSITION OF CELLULOSE.

Two laboratory peat fermentation experiments were started with the object of studying the possibility of bringing about rapid decomposition of cellulose in samples of Alberta peat. The acid surface sample of Carnwood peat, and the nearly neutral surface and subsurface samples of Winterburn peat were used.

Cultures (1) without added fertiliser, (2) with nitrogen, phosphorus and potassium, and (3) with nitrogen, phosphorus, potassium and lime, were prepared. Nitrogen and phosphorus were added in the form of ammonium phosphate ($(\text{NH}_4)_2\text{HPO}_4$). Potassium was added in the form of potassium hydroxide (KOH), in order to decrease the acidity of the acid peat, and lime was added in the form of powdered calcium carbonate (CaCO_3). The fertiliser salts were added in relatively large quantities since nitrogen, phosphorus and potassium were each added at the rate of about 2 gm. and calcium carbonate at the rate of 4 gm. per 100 gm. water-free peat.

The peat was coarsely ground and placed in 750 c.c. culture flasks at the rate of 25 gm. water-free peat in each flask. The optimum quantities of moisture and the desired salts were added, and the cultures were fermented at a temperature of approximately 30°C . Aeration was provided by plugging the flasks with cotton-wool, and by stirring the contents with a glass rod every second day. Moisture was added as required to maintain optimum conditions.

After several days' incubation the cultures were tested for hydrogen-ion concentration, and gave the following values: Carnwood surface peat with moisture only, *pH* 4.0, and with moisture and salts, *pH* 6.5; Winterburn surface peat with moisture only, *pH* 6.5, and with added salts *pH* 8; Winterburn subsurface peat with moisture only *pH* 6.5, and with added salts, *pH* 7.5.

At the end of 50 days the percentages of cellulose in the incubated samples were determined (Table IV). In the case of the Carnwood surface peat an appreciable loss of cellulose had occurred in the cultures to which fertiliser salts had been added, and especially in the cultures to which lime had been added. In the cases of the Winterburn surface and subsurface samples the losses, if any, were within the experimental error of the cellulose determinations.

Table IV.

Peat incubated for 50 days with abundance of salt under moist conditions at a temperature of approximately 30° C.

Treatment	Carnwood surface peat cellulose (minus ash) %	Winterburn surface peat cellulose (minus ash) %	Winterburn subsurface peat cellulose (minus ash) %
Original peat, unincubated	26.0	33.5	5.1
Incubated with moisture only	26.5	33.7	5.4
Incubated with moisture + N + P + K	22.0	33.3	5.2
Incubated with moisture + N + P + K + Ca	19.9	—	—

Table V.

Plate counts of the bacteria in the different cultures of Carnwood surface peat at the end of the 50-day incubation period.

Treatment	No. per gm. of original peat
Incubated with moisture only	29,000,000
Incubated with moisture + N + P + K	66,000,000
Incubated with moisture + N + P + K + Ca	70,000,000

At the end of 50 days the Carnwood peat cultures were plated out on Thornton's medium which restricts the spreading of individual colonies and consequent crowding out of other colonies(11). It will be observed (Table V) that the bacterial numbers were more than doubled by the addition of the fertiliser salts, and, with longer periods of incubation it is likely that much greater differences in loss of cellulose could be measured, corresponding more closely with the relative numbers of bacteria.

A second experiment was started with the object of studying the possibility of bringing about rapid decomposition of cellulose in Alberta

262 *Study of Composition and Utilisation of Alberta Peats*

peat. The acid surface sample of Spruce Grove peat, which contains a high percentage of cellulose, was used.

Cultures (1) without added fertiliser, (2) with nitrogen, phosphorus, potassium, magnesium and sulphur, and (3) with nitrogen, phosphorus, potassium, magnesium, sulphur and lime were prepared. Nitrogen and phosphorus were added in the form of di-ammonium phosphate $((\text{NH}_4)_2\text{HPO}_4)$. Potassium was added in the form of potassium hydroxide (KOH), in order to decrease the acidity of the acid peat. Magnesium and sulphur were added in the form of magnesium sulphate $(\text{MgSO}_4 \cdot 7\text{H}_2\text{O})$, and lime in the form of powdered calcium carbonate (CaCO_3) . The fertiliser salts were given in relatively large quantities, the various elements being added at the following rates per 100 gm. water-free peat: nitrogen 2.0 gm., phosphorus 2.2 gm., potassium 2.5 gm., magnesium 0.5 gm., sulphur 0.6 gm. and calcium carbonate 4.0 gm.

The peat was ground coarsely and placed in 750 c.c. flasks at the rate of 25 gm. water-free peat in each flask. The desired salts were added and the peat was saturated with moisture. Some aeration was provided by plugging the flasks with cotton-wool, and by stirring the contents of each flask every second day with a glass rod. Moisture was added as required to maintain a saturated condition. The cultures were incubated at the relatively high temperature of 55°C . for a period of 19 days.

At the end of this period the cultures were analysed for cellulose, corrections being made for losses in weight of peat (or gains due to the addition of salts). The percentages of cellulose in the peat before and after incubation are shown in Table VI. Losses of cellulose occurred in all of the incubated samples. The largest loss, amounting to nearly 30 per cent. of the cellulose originally present, occurred in the culture to which lime was added in addition to the other nutrient salts.

Table VI.

Spruce Grove surface peat incubated for 19 days with abundance of salt under very moist conditions, at a temperature of 55°C .

Treatment	Cellulose (minus ash)
Original peat, unincubated	46.9
Incubated with moisture only	40.7
Incubated with moisture + N + P + K + Mg + S	38.6
Incubated with moisture + N + P + K + Mg + S + Ca	33.1

From these experimental results it is clear that the peat cellulose is not nearly as readily decomposable as the filter paper cellulose used in the experiment discussed in an earlier section of this paper. The resistance

of the peat cellulose to decomposition may be due to protection of cellulose by lignin and other substances, and greater resistance to decomposition of peat cellulose as compared to filter paper cellulose.

DISCUSSION.

The cellulose fermentation experiment described in this paper showed very clearly that under the conditions of this experiment (and probably under any conditions) the three elements commonly applied in the form of mineral fertilisers in farm practice will not produce rapid decomposition of filter paper cellulose. Even after 107 days these elements had produced very little decomposition, whereas decomposition was rapid in the cultures to which the other "essential" elements were added. The importance of adding all "essential" elements to the cultures when testing the ability of different micro-organisms to bring about decomposition of purified forms of organic matter, such as filter paper cellulose, or when studying the course of the decomposition of purified forms of organic matter, is therefore apparent.

The numbers of ammonifying bacteria in the cellulose fermentation cultures, determined by the plate count method, increased with each additional "essential" element or group of "essential" elements. It is suggested, however, that the rapid decomposition of cellulose in the cultures to which all "essential" elements were added was brought about mainly by fungi, because repeated attempts to determine the numbers of cellulose decomposing bacteria in these cultures by plate count methods were unsuccessful. The ammonifying bacteria would probably be very largely concerned with the secondary stages of the decomposition.

Different horizons or layers of the Alberta peats studied differ greatly in colour or stage of decomposition, and in reaction of pH value. The surface samples usually contain less ash than the deeper samples because the surface samples are less decomposed. The subsurface layer usually contains about twice as much nitrogen as the surface layer because carbon is lost more rapidly than nitrogen when newly formed peat decomposes. The nitrogen content of the different samples varies more than the phosphorus content. Peats reclaimed for crop production are frequently deficient in available phosphorus, nitrogen and potassium. The calcium oxide percentages and the pH values indicate that the Carnwood and Spruce Grove peats require liming, and that the Winterburn and Stonyplain peats do not require liming, for satisfactory crop production.

264 *Study of Composition and Utilisation of Alberta Peats*

A decrease in cellulose content is usually accompanied by an increase in lignin, and this is to be expected if we assume that when plant residues decompose the cellulose decomposes much more readily than the lignin. Cellulose, lignin, and ash together nearly always make up about two-thirds or more of the weight of the peat, and the remaining fraction would be made up of a variety of substances.

Growth of oat seedlings and bacterial plate counts indicated that the fertility of Carnwood surface peat was not greatly increased or affected by the addition (about three to four months earlier) of ordinary application amounts of fertiliser salts. The experiment indicates that surface peats of this nature with high percentages of cellulose are not easily decomposed, and suggests that the process of reclamation for satisfactory crop production will require a period of years. However, if the fertiliser salts had been applied at the time of planting the seeds their effects upon plant growth might have been greater.

The laboratory experiments conducted with the object of studying the possibility of bringing about rapid decomposition of peat cellulose showed that under favourable conditions, including a sufficient supply of nutrient salts, peat cellulose can be decomposed. It is not nearly as readily decomposable as filter paper cellulose, but it is doubtful if the peat will form a satisfactory soil for common crops until the excess of cellulose has been decomposed. It is well known that the addition to ordinary mineral soils of excessive quantities of organic matter rich in cellulose, such as wheat straw, will reduce the fertility of the soil until the excess of cellulose has been decomposed.

It is suggested that the possibility of utilising Canadian peats which contain an exceptionally high percentage of cellulose for commercial purposes such as production of paper, alcohol, acetic acid, etc., should be investigated.

SUMMARY.

1. The three elements commonly applied in the form of mineral fertilisers in farm practice did not produce rapid decomposition of filter paper cellulose in incubated cultures, whereas the addition of all "essential" elements produced rapid decomposition.

2. Fungi appeared to be more important than bacteria in the decomposition of the filter paper cellulose.

3. The numbers of ammonifying bacteria in cellulose fermentation cultures increased with each additional "essential" element or group of "essential" elements.

4. Different horizons or layers of the Alberta peats studied differ greatly in colour or stage of decomposition and in reaction or pH value.

5. The surface samples of peat usually contain less ash than the deeper samples.

6. The nitrogen content of the different samples varies rather widely, and the subsurface layer usually contains about twice as much nitrogen as the surface layer.

7. The total phosphorus content of the different samples does not vary as much as the nitrogen content.

8. The calcium oxide percentages and the pH values indicate that the Carnwood and Spruce Grove peats require liming and that the Winterburn and Stonyplain peats do not require liming for satisfactory crop production.

9. The cellulose content of the peats varies from none to about 47 per cent. of ash-free cellulose, and the lignin from about 20 to 49 per cent. A decrease in cellulose content is usually accompanied by an increase in lignin.

10. Cellulose, lignin, and ash together nearly always make up about two-thirds or more of the weight of the peat. Nitrogenous organic matter would account for about 3-16 per cent., and petroleum-ether-soluble material for only 1 per cent. or less of the total peat.

11. Growth of oat seedlings and bacterial plate counts indicated that the fertility of Carnwood surface peat was not greatly increased or affected by the addition (about three to four months earlier) of ordinary applications of fertiliser salts.

12. At the end of an incubation period of 50 days appreciable losses of cellulose had occurred in the Carnwood peat cultures to which an abundant supply of fertiliser salts had been added; and bacterial numbers were increased by the addition of fertiliser salts. In the case of the Winterburn peat the losses of cellulose, if any, were within the experimental error of the determination.

13. After nineteen days' incubation at a relatively high temperature (55° C.) all of the cultures of Spruce Grove peat showed loss of cellulose, the largest loss occurring in the culture to which lime was given in addition to an abundant supply of the other nutrient salts.

ACKNOWLEDGMENT.

The writer wishes to acknowledge a Carnegie Corporation Grant from the Universities Bureau of the British Empire which enabled him to come to Britain and carry on the work reported in this paper.

266 *Study of Composition and Utilisation of Alberta Peats*

The work has been carried on mainly at the Rothamsted Experimental Station in the Department of Mr D. Ward Cutler. The writer wishes to thank Sir John Russell for placing the facilities of the Station at his disposal, and to acknowledge the very useful advice obtained from various members of the Rothamsted staff.

REFERENCES.

- (1) ALWAY, F. J. and NYGARD, I. J. (1927). Differentiation between lime-deficiency and acidity in the case of peat soils. *Proceedings and Papers of the First International Congress of Soil Science*, vol. II.
- (2) BEDFORD, R. H. (1919). The decomposition of peat measured by carbon dioxide evolved. *Sci. Agric.* ix, 8, pp. 522-32.
- (3) DU TOIT, H. M. S. and PAGE, H. J. (1930). Studies on the carbon and nitrogen cycles in soil. III. The formation of natural humic matter. *Journ. Agric. Sci.* xx, 478-88.
- (4) HAANEL, B. F. (1926). Peat, its manufacture and uses. *Canada Dept. of Mines Branch Pub.* No. 641.
- (5) JENKINS, S. H. (1930). The determination of cellulose in straws. *Biochem. Journ.* xxiv, 1429-32.
- (6) JENSEN, H. L. (1931). The microbiology of farmyard manure decomposition in soil. II. Decomposition of cellulose. *Journ. Agric. Sci.* xxi, 81-100.
- (7) LEWIS, F. J., DOWDING, E. S. and MOSS, E. H. (1928). The vegetation of Alberta. II. The swamp, moor and bog forest vegetation of Central Alberta. *Journ. Ecology*, xvi, 19-70.
- (8) NORMAN, A. G. (1931). The biological decomposition of plant materials. IV. The biochemical activities on straw of some cellulose-decomposing fungi. *Ann. Appl. Biol.* xviii, 244-59.
- (9) OGG, W. G. (1932). The Macaulay Institute for Soil Research. *Scottish Journ. Agric.* xv, January.
- (10) SCHWALBE, H. (1925). Eine neue Methode zur Bestimmung des Lignins. *Papier-Fabrikant*, xxiii, 174-7.
- (11) THORNTON, H. G. (1922). On the development of a standard agar medium for counting soil bacteria, with especial regard to the repression of spreading colonies. *Ann. Appl. Biol.* ix, 241.
- (12) WAKSMAN, S. A. and STEVENS, K. R. (1929). Contribution to the chemical composition of peat. IV. Chemical studies of highmoor peat profiles from Maine. *Soil Science*, xxvii, No. 5, pp. 389-98.

(Received August 16th, 1933.)

SOME BIOLOGICAL AND ECONOMIC ASPECTS OF THE GALL MIDGES

By H. F. BARNES, M.A., PH.D.

Rothamsted Experimental Station

Part I.—Introductory and Biological

INTRODUCTION

THE gall midges (*Cecidomyidæ*) comprise a family of delicate flies, second to none in their variety of form and abundance. Small in size, varying from under 1 mm. to about 6 mm. in length, they may be most readily recognised by their antennæ and the simple venation of their wings. Most of them in their larval stage cause malformations or galls on plants and hence the name that has been applied to them.

Historically they rank as old stagers, Pliny having described a gall formed by one of them on beech leaves. Further references to their galls can be found in the literature of the sixteenth, seventeenth and eighteenth centuries, but with few exceptions no mention is made of the insects producing these galls. Réamur (1736–40) was the first to describe some larvæ and Linnæus (1758) was the first to describe one of the perfect insects. Other old writers, including De Geer, Schranck, Geoffroy and Fabricius, described other kinds. In 1803 Meigen recognised them as a distinct group and created the genus *Cecidomyia* (*κηκιδος* gall, *μυια* fly) to include all such forms as were then known. Notwithstanding the initial monographs of Bremi (1847), H. Loew (1850), and Winnertz (1853), and a succession of many writers such as Laboulbène, F. Loew, Meinert, B. Wagner, Karsch, Mik, Riley and Lintner, the knowledge of gall midges was very fragmentary until the last forty years. Attention had been focussed on the galls, and their producers had been largely either forgotten or ignored.

Then arose an international triumvirate—Rubsamen, Kieffer and Felt—to whom the insect was more important than the gall and whose names will for ever be associated with the knowledge of gall midges. Tavares should also be linked with the big three

of modern times. These four workers, with the possible exception of Kieffer (see his *Monographie des Cecidomyides d'Europe et d'Algérie*, 1900), turned their thoughts chiefly if not wholly towards the systematics of the Cecidomyidæ. Their work, represented by series of monographic papers, is of the utmost importance. They have laid the basis of a lasting classification. Besides these workers, several students of the group have paid special attention to such forms as are of outstanding economic importance, for example the gall midges of cereals were studied by Marchal; and all the time there have been numerous investigators who have been describing new malformations due to these insects. The standard works on galls are those of Houard, who has written a series of volumes dealing with the galls of Europe, Asia and Oceania as well as Central and South America, and that by Felt on insect galls of America.

Geologically, the oldest species known is one of the Secondary period found in English Purbeck. Several species which existed in the Tertiary period have been found in Baltic amber (Eocene) and in America representatives have been discovered in Oligocene beds and in Pleistocene swamp deposits. In the Quaternary period, a species has been found in copal of Africa.

Geographically, the distribution of these insects is world-wide, a representative being recorded even from New Caledonia. But while the European and North American fauna are comparatively well known, those of Australia, New Zealand, India, Africa and many other places have been very incompletely studied.

Numerically, the family is large. The number of species now described is about 3,500 belonging to about 490 genera. The proportion of genera containing only one species is considerable and probably more knowledge of the family will enable better groupings to be made in the future. It is perfectly safe to assume however that there are many species awaiting discovery. Only about 30 species are recorded as occurring in New Zealand, about 100 each in Australia and India and about 150 from Africa as compared with 900 from the United States of America and about 500 from Great Britain.

It is the purpose of this article to illustrate the diversity of habit exhibited by the gall midges and their economic importance and to follow this by an account of the various methods which have been found effective in their control. The diversity in their structure can be appreciated by reading the works of Felt (*Key to Gall Midges*, N.Y. State Museum Bulletin 257, 1925) and Kieffer (*Genera Insectorum*, fascicule 152, 1913).

BIONOMICS

MORPHOLOGICAL VARIATION IN ADULTS CORRELATED WITH FOOD OF LARVÆ.—The antennæ of the adult midges present an extraordinary range in development and structure. For example, in some species they have but six segments while others have been described with as many as thirty-six and forty-one segments. In the generalised forms there are fourteen segments, but as Felt has stated there seems to be some connection between the size of the insect and the number of antennal segments. A recent exhaustive study¹ has revealed the most interesting fact that the number of antennal segments in the adult midge is in some cases correlated with the food of the larvæ. By causing the parent midges to lay more eggs than usual in one position, thus overcrowding the resultant larvæ, and also by causing the midges to oviposit on rather distasteful plants, it has been possible to produce small adults with a low number of antennal segments. On the other hand by allowing the midges to oviposit under ideal conditions, considerably larger midges possessing a distinctly larger number of antennal segments have been obtained. Breeding from such individuals under bad conditions immediately resulted in the production of small midges with a low number of antennal segments. Such variation in antennal numbers obtains only in certain species which do not have the generalised numbers of segments, whereas food affects the size in all species. This may be an indication of the plasticity of certain species and subsequently may throw light on the evolution of species.

UNISEXUAL FAMILIES.—Normally reproduction in the gall midges is sexual and although parthenogenesis has been claimed to occur this has never been substantiated. In a few instances, however, unisexual families are the rule. In addition to reproduction by the adults pädogenesis, which is the multiplication of individuals by larvæ, takes place. But this phenomenon will be discussed later.

The result of sexual reproduction as suggested by the chromosome theory is that both sexes are produced in approximately equal numbers. Nevertheless it has been shown by Hindle, when dealing with the common louse of man *Pediculus humanus*, and by Metz, whose studies on the fungus gnats of the genus *Sciara* deserve far greater attention than they have been given, that some insects although reproducing sexually give rise to four types of broods. These types are (1) entirely male, (2) entirely female, (3) male

¹ *Zoo. Soc. London* for 1932, 1932, 323-34.

and female with the number of males predominating, and (4) male and female with the number of females predominating. Metz has found in *Sciara* that 25 per cent. of the progeny of a single female-producing female when mated will produce all-male families, 25 per cent. will produce all-female families, while the remaining 50 per cent. produce families in which both sexes are represented. Of these mixed families one-half have the males in the majority and the other half have the females. The male does not directly affect the sex of the offspring of the female with which pairing takes place. This at one time was presumed to indicate that there was selective elimination of the sperm by the female. But Metz has since shown that there exists a much more subtle and complicated state of affairs in the chromosomal mechanism. The possibility of non-fertilisation of some of the eggs does not enter into the question. This makes the problem of so much interest, especially as in some other insects the same four types of families have been observed. For instance, Bald and Davidson have obtained all-male families, all-female families and mixed families in *Frankliniella insularis*, a thrips. But in this case the all-male families were derived from virgin females and presumably the males in the mixed families arose from unfertilised eggs as the all-female families were derived from fertilised females.

This production of unisexual families, as they are called, has also been found to be the rule in about six species of the genus *Rhabdophaga* and one species of the genus *Thomasiniana* among the gall midges.¹ Here again individual females of an all-female family can give rise to any one of the four types of family. The outstanding feature of such sexual reproduction is that it usually precludes brother and sister mating. The result of this should be beneficial to the race as unwanted injurious recessive characters will not appear so frequently.

In other species, while unisexual families are not the rule, there is a significant departure from the 1 : 1 ratio irrespective of percentages. In still other species the males appear to be in the minority as a normal occurrence giving a sex ratio of about 25 : 75.

FLUCTUATION IN INSECT NUMBERS.²—For a variety of reasons the gall midges are particularly suitable for population studies. In the first place it is quite possible to obtain large numbers of a particular species with a minimum amount of trouble. For example, from 120 heads of Meadow Foxtail grass from Dorset over 21,000 larvæ of *Contarinia merceri* have been obtained. Another reason

¹ *Jl. Genetics*, 24, 1931, 225-34.

² *Jl. Animal Ecology*, 1, 1932, 12-31, and 191-2; *ibid.*, 2, 1933, 98-108.

is that while some species are univoltine, others have several broods a year. However, it is only of recent years that such studies have been made and already several interesting results have been obtained.

The rapidity with which a pest can increase and decrease has been demonstrated; in the space of a year or one generation the numbers of *Contarinia tritici* larvæ in 500 ears of wheat rose from 2,200 to about 19,300, and in another year a decrease of the same magnitude has been observed.

There are indications that weather conditions are of greater influence than natural enemies and parasites. In one year when the wheat ears burst their sheaths a fortnight earlier than usual, the midges emerged about three weeks before their normal date. As the midges lay their eggs on the newly emerged wheat-ears and are short lived, one would have expected that in such a season there would be a great reduction in the infestation of the crop. This is exactly what happened. In the case of *Sitodiplosis mosellana*, one of the wheat-blossom midges, the number of larvæ found in 500 ears of wheat was just over 300 compared with about 3,000 the previous season. No such violent reduction in numbers has yet been found to be directly due to the action of parasites.

In another case, owing probably to the differential effect of weather on the midges and their parasites which caused the parasites to emerge before the host midges, an outbreak of the midge has been noted. From an initial population of nearly 1,600 midges, obtained from 100 heads of Meadow Foxtail grass, the population rose to nearly 5,000 in the same sized sample. The numbers of parasites likewise fell enormously. A similar increase has been seen in another species of gall midge, *Rhabdophaga heterobia*. This midge is multivoltine, and usually about 1,500 midges have been reared from 500 galls as well as about 1,500 Hymenopterous parasites. In one particular year, however, about 2,800 midges were bred and only about 400 parasites. Thus while the total insect population remained about constant the parasitism fell from about 50 per cent. to about 13 per cent. In another year owing to drought affecting the larval food supply the total population fell from the 3,000 mark to about 1,000, but in this case the relative numbers of midges and parasites were not altered.

Another peculiarly interesting fact has arisen regarding the parasitism of a multivoltine species, *Dasyneura pyri*. Samples of full-grown larvæ were obtained periodically from a very restricted area in Devon. For two years not a single parasite was recorded; in the subsequent four years parasites appeared but not in every generation of the midges. Three times were they present in the

overwintering generation and three times were they present in the second generation of the particular year, but not once were they recorded from the first generation of a year. Is this an instance where the parasite has not yet become perfectly adjusted to its host's life-cycle?

PARASITES AND ENEMIES.—Knowledge of the Hymenopterous parasites and other enemies of the Cecidomyidæ is still fragmentary, but Marchal has studied several parasites in detail including the Proctotrupid *Inostemma piricola* and the Platygaster *Trichacis remulus*. Birds, such as tits which attack the galls of *Rhabdophaga heterobia*, and bugs belonging to the family *Anthocoridae*, for example *Anthocoris nemorum* which sucks the larvæ, pupæ and emerging adults of *Dasyneura pyri*, are to be numbered among the enemies. A vast field of research still lies open in this direction.

PÆDOGENESIS.—The production of larvæ by mother-larvæ, as they are called, has been known for a long time to occur in the subfamily *Heteropezinae* of the Cecidomyidæ, but now this phenomenon has been shown to occur in addition in another subfamily, the *Lestremiinae*.

Harris¹ and Gabritschevsky,² two recent workers on pædogenetic forms, have shown that the larvæ of *Miastor* and *Oligarces* have three forms. There are (a) typical white pædogenetic larvæ without a sternal spatula or breastbone (anchor-process); (b) yellow pædogenetic larvæ without a sternal spatula, the significance of these forms is obscure; and (c) pupa-forming larvæ which possess a sternal spatula and which develop into adults. The adults mate normally and produce the pædogenetic larvæ. The occurrence of these three larval forms has been shown to depend on environmental conditions. Only one sex is derived from one mother-larva.

LARVAL FOOD HABITS.—The larvæ of gall midges exhibit an amazing diversity in their food habits and a close adaptation to their particular food, both of which typify the high degree of specialisation reached in the family. They can be primarily classified into two groups: (a) the phytophagous forms and (b) the zoophagous forms.

PHYTOPHAGOUS LARVÆ.—The great majority of gall midges come into this group which can be subdivided into those forms

¹ *Psyche*, 30, 1923, 95–101; loc. cit., 31, 1924, 148–54; *Biol. Bull.*, 48, 1925, 139–44; *C. R. Soc. Biol. Paris*, 88, 1923, 256–8; *Proc. Nat. Acad. Sci.*, 9, 1923.

² *Bull. Soc. Ent. France*, 1928, 75–9; *Bull. Biol. Fr. Belgique*, 62, 1928, 478–524; *Archiv. Entw. Mech. Org.*, 121, 1930, 450–65.

which cause galls, those forms which live in galls but do not cause them, and those forms which live on plants but neither cause galls nor live in them.

Although almost every part of the plant, including the root, seems liable to gall formation, most of the galls are formed on the soft parts such as the buds, leaves and fruit. Indeed Felt has estimated that 70 per cent. of the American gall midges form such galls, while only 30 per cent. form galls on the harder parts such as the stems. In addition, while most plants maintain one or more kinds of gall midge, there are striking groupings of allied species of midge and allied plant species. For example, species of the genus *Rhabdophaga* are, with few exceptions, restricted to the genus *Salix*. This is further evidence of the great plasticity of the gall midges in that certain genera have adapted themselves to various host plants.

There are relatively few forms which live in galls made by other insects but do not cause them. Most of these are to be found in the galls of other Cecidomyidæ; but several live in galls produced by Cynipids, others live in galls produced by Muscid flies, and at least one inhabits the galls made by a beetle. The exact mode of feeding of these larvæ is not clear, but they are certainly not predaceous.

The second largest group of the gall midges as a whole feed on plant tissue but do not inhabit galls. Many live in the florets of grasses and cereals, feeding on the developing seed. Other forms feed on resin, under the bark of trees, in decaying vegetation and on newly felled timber. It is of interest to note here that in recent years there has been an isolated case of gall midge larvæ being so numerous on the bodywork of a new motor-car (*i.e.* coming out of the wood) that the firm thought it desirable to burn the coachwork and presumably compensate the owner. Another group feeds on rusts and other fungi. There is also a coprophilous group which feeds on the excreta of various fly and beetle larvæ under bark and in old birds' nests.

ZOOPHAGOUS LARVÆ.—These gall midges can be conveniently divided into those which are internal parasites and those which are predaceous. In the first group the best known example is *Endaphis perfidus* whose larvæ live inside an aphid of Sycamore leaves. There is also another midge whose eggs are laid on the wings of the apple sucker *Psyllia mali* and whose larvæ are internal parasites of the *Psyllia*. There is need here for some detailed morphological studies as to the structural adaptations involved. The second group comprises a number of forms which are pre-

daceous on other gall midge larvæ, those which feed on mites and red spider, and those which feed on greenfly (Aphididæ), scale-insects and mealy-bugs (Coccidæ), white-fly (Aleyrodidæ) and thrips (Thripidæ). Recent compilations have shown that there have been described about 50 species whose larvæ are known to feed on aphids, about 40 species on mites, about 40 species on scale insects and two on whitefly, while at least two are known to eat thrips.

CHOICE AND RESTRICTION OF HOST PLANT.—It is well known that gall midges as a rule are very limited in their range of host plants and are intimately associated with them. While some authorities have claimed that certain midge species live on a number of plant species, there are many indications in the opposite direction. These claims have usually been based on similar gall formation, or the larvæ found in the galls or in a few cases only on the adult midges. Any one who has had any experience of the gall midges as a group can at once point out the danger attendant on any one of these methods of identification, if used alone. The gall is the reaction of the plant to the presence of the insects and can vary considerably as will be shown later. No one would identify a midge species on larvæ unless previous experience had indicated that it could not possibly be mistaken for any allied species. And lastly midge species are exceedingly difficult to separate even in the adult stage. The more individuals one examines, the wider the variation seen. This applies to any one dealing with hundreds of either sex, not a paltry five or six.

The wheat-blossom midges have been found by experiment to be able to oviposit and live successfully on other plants besides wheat, *e.g.* *Contarinia tritici* on Couch grass, Slender Foxtail grass and Rye. Other host plants are also recorded for this species. Yet other species of the genus *Contarinia* seem restricted to one species of grass. Thus it appears possible that there are certain plastic species able to live on several host plants while other species are evolving and becoming restricted to particular plant species.

Investigations on willow midges¹ have shown that one midge, *Rhabdophaga heterobia*, will attack all varieties of *Salix triandra* but will not form galls on *S. viminalis*, *S. purpurea*, *S. purpurea* x *viminalis*, *S. viminalis* x *triandra*, *S. alba* var. *vitellina*, *S. alba*, *S. cærulea*, *S. fragilis*, *S. nigricans*, *S. americana* nor on *S. repens*. Sometimes eggs are laid on some of these species but no galls result. Another species, *R. terminalis*, lives on two willow species

¹ *Ann. App. Biol.*, **18**, 1931, 75–82; *loc. cit.*, **19**, 1932, 243–52; *The Times*, February 6, 1933; and unpublished data.

S. cærulea and *S. alba* var. *vitellina*, but chooses the former when available. It will not live on *S. triandra*, *S. viminalis*, *S. purpurea* nor *S. viminalis* x *triandra*. The possibility of midge strains must not however be ignored, especially in view of Painter's work mentioned below.

A few studies have recently been made with reference to the varietal resistance of plants to particular midge species. For example, Painter, Salmon and Parker¹ have studied the resistance of wheat to the Hessian fly. Among interesting data five characteristics which affect the resistant qualities are discussed. These are (1) a decided difference in the number of flies which develop on the several varieties, (2) a kind of tolerance permitting the fly to develop without material damage to the plant, (3) the ability of some varieties of wheat to develop fly better and faster than other varieties, (4) the ability of wheat to develop tillers after infestation, and (5) the stiffness of straw in relation to Hessian fly damage. Painter has also found distinct biological strains of Hessian fly, one in the hard-wheat belt of central and western Kansas and one in the soft-wheat belt area of eastern Kansas.

GALL AND INSECT SPECIES.—Students of galls often identify gall midges by the kind of gall produced. There are many dangers in such a procedure. The state of growth of the plant at the time of oviposition by the insect and immediately subsequent to this has a great influence on the gall produced. For example, *Rhabdophaga terminalis* lays its eggs on the terminal buds of *Salix cærulea* and the terminal leaves remain in a curled and crinkled state instead of unfolding naturally. The gall is at first reddish but later turns black. Sometimes, however, when the development of the eggs is retarded by the inclement weather more than the growth of the plant, the shoot goes on growing away from the leaves on which the eggs were originally laid. In such cases these latter leaves open and the larvæ form blister-like galls along the midvein of the leaf.² In much the same way, the galls of *R. heterobia* vary from a type of gall which is merely a swollen lateral bud to the type in which it is a perfect rosette of small leaves.

A single species of gall midge frequently produces several distinct types of galls. The pea midge,³ *Contarinia pisi*, causes malformed flowers, clusters of leaves and flowers with the flower-stalks absent or nearly so, and the larvæ also live inside the pods.

Rhopalomyia hirtipes,⁴ an American species, produces an apical

¹ Kansas Agric. Expt. Station, Tech. Bull. 27, 1931.

² Ann. Appl. Biol., 19, 1932, 246-7. ³ Jl. Min. Agr., 34, 1927, 159-61.

⁴ N.Y. State Mus. Bull., 257, 1925, 24.

gall on stunted shoots of *Solidago* but it also causes a subterranean gall on the buds from the root stocks.

Furthermore several species of gall midges can be reared from the same gall and from the same kind of gall on one or more plants. In short then, it is hazardous in many cases to identify gall midge species using the galls or the galls and larvæ only until much more biological work has been done on the midges themselves and the interrelationships of plant reactions and midge attack.

Such then are the bionomics of the gall midges.

Part II.—Economic Importance and Control

ECONOMIC IMPORTANCE

One would expect that a large family of flies, in which “adaptive radiation” is so pronounced, would be of considerable interest to the economic entomologist. Such is the case. The well-known Hessian fly is perhaps the best example because of the immense loss it has caused in the past to wheat growers in the United States of America. The pear midge, *Contarinia pyrivora*, whose larvæ destroy young pears, is better known in this country. The variety of crops which suffer from gall midge attacks is almost infinite: among field crops may be mentioned swedes and cauliflowers; wheat and sorghum among cereals; pear, cranberry and tomato among fruit; violets and newly budded roses among horticultural plants; grapes, chrysanthemum and *Hydrangea* among greenhouse plants; and box among ornamental shrubs. Among willow growers both for the basket and the cricket-bat industries, midges are again responsible for losses. Hop growers also suffer from the “Strig maggot.” Seed production, especially that of red clover and grasses such as meadow foxtail, cocksfoot and timothy, is seriously affected.

Besides the many injurious species of gall midges there are some of beneficial importance although they have only received scant attention up to the present. The prickly pear midges do so much damage that Felt has stated that they would be regarded as beneficial in an area where these plants are undesirable. Felt has also stated that *Dasyneura gibsoni*, whose larvæ live in thistle-heads, is regarded as a useful check on the spread of thistle seed. In addition, many of the predaceous gall midges feed on injurious insects and mites and without doubt form a useful link in the faunal “chains” they frequent. As an example *Therodiplosis persicæ*, whose larvæ feed on red spider in glass-houses, can be mentioned.

CONTROL MEASURES

The chief methods of controlling insect pests are (1) cultural, (2) biological, (3) physical such as the use of heat, (4) chemical, (5) trapping such as grease banding, and (6) legislation. The first and last of these may be considered as preventive, while the fourth can only at best be palliative. Biological control may be either preventive or palliative. While there is a very real difference between the prevention and the cure of insect outbreaks, it is sometimes difficult to draw hard and fast lines of distinction between the methods of control. A measure may be partly preventive and partly remedial.

CULTURAL METHODS.—Such methods of control are by far the most important in thwarting the ravages of gall midges and it will be seen that with few exceptions any control measures known to be useful against these insects are cultural in one form or another. Nearly all such methods aim at destroying the normal sequence of events in the life history of the pest and its food or host plant.

The use of clean seed is fast becoming more and more a *sine qua non*. It is almost certain that one of the meadow foxtail midges, *Dasyneura alopecuri*, originally a European pest, was introduced into New Zealand, where it is now a pest, through infected seed. Other midges that could be distributed with the seed are the pea midge (*Contarinia pisi*), the red clover midge (*Dasyneura leguminicola*) and the pod midge (*D. brassicae*).

The date of sowing is very important in the case of attacks by at least one midge. A crop should be sown early or late according to the life history in the particular locality of the midge to be avoided. In America the sowing of wheat is delayed until the "safety" or "fly-free" date, which is when the main flight of the Hessian fly is over. This method of control is based upon the fact that midges are short lived and that if a crop be sown after the main flight of a brood the crop should escape most of the injury to which it is liable under normal conditions. The "safety" date originally had to be determined in the first place separately for each locality by direct observation. Such observations have been eliminated to some extent by the judicious use of the bioclimatic law which was developed by Hopkins. This states that all other conditions being equal, the variation in the date of a periodic event in the seasonal activities of a plant or animal in America, north of Mexico, is at an average rate of four days for each degree of latitude, five degrees of longitude and 400 feet of altitude—earlier southward, westward and descending

in the spring and early summer, and later in the reverse directions in late summer and autumn. It has been shown that wheat sown before the "fly-free" date was heavily infested, but that when wheat sowing was delayed until after this date very little infestation occurred providing that there was no volunteer or self-sown wheat.

The time of harvesting has been shown to have a marked effect on infestation by certain midges. In order to grow a crop of red clover seed it is the practice to cut the first growth green and so starve out the larvæ of the red clover seed midges which are in the green heads of the clover. The subsequent growth of clover is free from midge attack as the direct result of this early cutting and so a good seed crop is obtained. Parallel to this cutting of a crop in order to kill larvæ already present, there is another method. This is to delay the crop flowering until the main flight of the particular midges involved is past and so prevent the larvæ ever getting on to the crop. This latter method has been shown to work in the case of the meadow foxtail midges. These midges normally prevent a good seed crop. By grazing or cutting the grass in order to prevent it from flowering until after the main flight of the midges, the loss of seed has been lowered in an experiment from 80 per cent. to 11 per cent.

Manurial treatment of a crop is sometimes beneficial in promoting rapid growth which results in a potential insect attack being frustrated. As an example of this it can be stated that whereas the normally slow growing cow-parsnip is very prone to attack by the midge *Macrolabis corrugans*, the cultivated parsnip is only rarely susceptible. In experiments although the midge oviposited time after time on the unopened leaves of the cultivated parsnip, only when the plants were developing slowly did the larvæ establish themselves. In every other instance the leaves uncurled rapidly and exposed the eggs of the midges to direct sunlight in which they perished. If the larvæ had had time to hatch the leaves would never have uncurled and so the larvæ would have survived.

The judicious use of manures and soil cultivation has also been shown to reduce the numbers of gall midge larvæ in the soil. For example there is the work of Klee¹ which has shown that top dressing in April preceded by ploughing in the autumn materially reduced attacks by the two wheat-blossom midges, *Contarinia tritici* and *Sitodiplosis mosellana*. Deep ploughing alone was distinctly beneficial but not so good as when followed by a top dressing.

¹ *Die Ernährung der Pflanze*, 18, 1932, 323-4.

A well-known practice to avoid pear midge attack is intensive cultivation between the rows of pear trees during the summer as a control against midge attack the following year. This cultivating and hoeing has on occasion¹ been highly successful. It is designed to destroy the larvæ in the soil after they have left the young pears.

The general cleanliness of crop is very important in the control of several midges, *e.g.* the Hessian fly, the swede midge and the wheat-blossom midges. Weeds must be discouraged as they provide alternative host plants for these midges. Self-sown wheat and crop remnants also help the midges in maintaining their numbers.

Rotation of crops can *a priori* be expected to keep down midge numbers and make things more difficult for them, especially in view of the fact that gall midges are not strong fliers.

The use of early or late varieties of plants as well as resistant varieties has been shown to be useful in the case of the Hessian fly, the wheat-blossom midges and willow midges. It is here that the entomologist must work hand in hand with plant breeders.

Trap crops have been recommended as a means of controlling the swede midge and the Hessian fly. Handpicking is always helpful when the crop is on a small scale, for instance in small orchards against pear midge attack.

There is an effective old method of preventing attack by *Thomasiniana oculiperda*, the red bud borer on bush roses. This midge is in the habit of laying its eggs in the slit between the inserted bud and the stock of newly budded plants such as roses and apples, thereby preventing the bud from taking. Thousands of roses have been lost by this happening. The method is to earth up the bushes so that the soil is above the level of the buds, in this way the adult midges are unable to lay their eggs. The soil is levelled again in the autumn after the buds have taken. This method has proved a distinct success in surface buddings.

The custom of spreading grease, such as vaseline,² over the tie as a precaution against the red bud borer when budding roses and apples may be considered as cultural in so far that it could and should be a normal practice in the procedure of budding. After the bud has been inserted, it must be tied in position and then a thorough coating of the grease should be applied. This prevents the midges from ovipositing. The actual reason for this avoidance by the midges is probably due to the stickiness, although

¹ *Ann. Rept. II Supplement, East Malling Res. Stat.*, 1931, 196-7.

² *Jl. Min. Agric.*, 37, 1930, 59-63.

it may be partly chemical. Such a method cannot be considered as trapping because the midges are not caught on the grease.

BIOLOGICAL CONTROL.—This method of pest control is as yet untried against gall midge attacks. But on the other hand there is a project under discussion whereby an attempt will be made to use a midge, *Therediplosis persicæ*, against red spider in glass-houses.

CHEMICAL CONTROL.—Straightforward applications of insecticides and fumigants have so far proved of little avail against insects of this family, with two exceptions. In America the boxwood leaf miner (*Monarthropalpus buxi*) is controlled by spraying with nicotine which penetrates the leaf tissue, and the chrysanthemum midge (*Diarthronomyia hypogæa*) is also controlled by fumigation and the use of sprays. But in the latter case the additional use of clean cuttings from the stock plants is most desirable.

LEGISLATION.—The use of legislation is essential in order to prevent the entry into new areas of proven pests. For example the boxwood leaf miner was introduced from Europe into the United States of America. Another instance was the recent outbreak of the chrysanthemum midge in a few English glass-houses. This was obviously a case of introduction from an infected area. Luckily it was prevented from establishing itself as a pest by the immediate spraying and burning of the infected plants whereby it seems to have been entirely wiped out.

To sum up, the most efficient and desirable ways of controlling and preventing gall midges doing great damage are those which can be grouped under the heading of Cultural Methods.

[FROM THE ANNALS OF APPLIED BIOLOGY, VOL. XX, No. 3,
pp. 498-519, AUGUST, 1933.]

[*All rights reserved.*]

A CAMBIUM MINER OF BASKET WILLOWS (AGROMYZIDAE) AND ITS INQUILINE GALL MIDGE (CECIDOMYIDAE)

BY H. F. BARNES, M.A., PH.D.

(Entomology Department, Rothamsted Experimental Station.)

(With Plates XXIX and XXX and 12 Text-figures.)

CONTENTS.

	PAGE
I. Introduction and methods	498
II. Identification and history	499
III. Morphology	501
(a) The egg	501
(b) The larva	501
(c) The pupa	509
(d) The adult, <i>Dizygomyza barnesi</i> sp.n. Hendel	509
IV. Distribution	510
V. Bionomics	510
(a) Emergence, mating and oviposition	510
(b) Damage	510
(c) Host plants	512
(d) Life cycle	512
VI. Parasites	513
VII. Inquiline gall midge, <i>Profeltiella dizygomyzae</i> sp.n.	514
(a) Description	514
(b) Distribution	516
(c) Bionomics	516
(d) Parasites	517
VIII. Summary	517
References	518
Explanation of Plates XXIX and XXX	518

I. INTRODUCTION AND METHODS.

THE following paper deals with the morphology and bionomics of an interesting Agromyzid fly which mines or tunnels in the cambium of basket willows. In addition, an inquiline gall midge is described for the first time and compared with another gall midge also new. The bionomics of this inquiline are given.

I am indebted to Mr T. Justin Cowley, who first showed me the larvae of this fly, for much assistance in this work. He has supplied me

both with material and information regarding the habits of the larvae. Mr H. P. Hutchinson, willow officer at the Long Ashton Research Station, has aided me considerably by discussing the problem and also supplying me with sets of the different willows used in the experiments. I am also grateful to Dr A. D. Imms, Dr C. B. Williams and Mr H. C. F. Newton for their help in diverse ways. Prof. F. Hendel, Mr J. E. Collin and Dr Ferrière have very kindly helped me with the identification of the fly and the parasites. In addition I should like to place on record my thanks to many correspondents, especially Mr H. McClelland of the Seale-Hayne Agricultural College. Mr V. Stansfield is responsible for the photographs.

Pupae were found in the first instance by sifting three sacks of soil taken from a space of 2 ft. square by 11 in. deep around an infested willow stub. From this amount of soil seven pupae were obtained. Larvae were collected by cutting rods in an infested area in July, August and September and then peeling the rods in the laboratory. Adult flies were caught in willow beds at Batford at the end of May and in early June.

For mating and oviposition, the flies were placed in glass muslin-covered cylinders which were stood over tubes containing willow shoots in water. When it was desired to breed from the flies they were put into muslin cages which had been placed over growing willow plants in 10–12 in. pots in an unheated greenhouse. In this way the complete life cycle was followed.

Some larvae were mounted directly in De Faure's fluid, others after being killed in boiling water and cleared with potash were stained with Ziehl's carbol-fuchsin and mounted in balsam.

II. IDENTIFICATION AND HISTORY.

The Agromyzid in question belongs to the genus *Dizygomyza* Hendel (subgen. *Dendromyza* Hendel) and is very closely allied to the two species *carbonaria* Zett. and *cambii* Hendel. The following notes are taken from a letter written by Dr Hendel:

"The four specimens (♂ ♀) kindly sent to me by Dr Barnes belong to the genus *Dizygomyza* Hendel (subgen. *Dendromyza* Hendel). The synopsis of the species in Hendel's Agromyzidae, p. 18 (in Lindner's *Die Fliegen der palaearktischen Region*, No. 59, 1931) runs to number 9 a. Neither *Dizygomyza cambii* Hend., nor *carbonaria* Zetterstedt *sensu* Hendel can be the same species as the fly of Dr Barnes, for both lack the postero-dorsal bristles on mid-tibiae.

"The single specimen of *Dizygom. cambii*, bred from *Salix* twigs by Prof. de Meijere in Holland has not haired eyes; but it has 6–7 orbital-

500 *A Cambium Miner of Basket Willows (Agromyzidae)*

bristles on the front and 10–11 irregular rows of acrostichal hairs between the dorso-central bristles on the mesonotum.

“*Dizygom. carbonaria* (Zett.) Hend. has the third *dc* bristle in the middle between the second *dc* and the suture of the mesonotum, while the third dorsocentral bristle of *cambii* and of the fly of Dr Barnes is distinctly approached to the mesonotal suture.

“Barnes’ fly has the haired eye of *carbonaria*, but the position of the *dc* bristles as in *cambii*.

“Only an examination of Zetterstedt’s types of *Agromyza carbonaria* in Lund may make a correct determination of these species possible.”

Dr Hendel has very kindly written a description of this fly¹ for me and this is included in section III(*d*) of this paper.

The history of cambium miners is limited and dates back to 1868 when Ratzeburg described some larvae found in the wood of birch trees in 1853 as *Tipula suspecta*. Previously Hartig in 1851 had described the damage². For some time, however, writers considered the “fleck marks” or “medullary spots” as one of the specific characters of forest trees. However, Kienitz (1883) made an important addition to the literature and came to the conclusion that the medullary spots of various deciduous trees were occluded channels which had been mined in the cambial cylinder by insects. One of his plates is reproduced as fig. 3 on Plate XXX. Grossenbacher (1910) gave a useful summary of the literature on the subject, as did also Brown (1913) and Record (1911). Nielsen (1906 *a* and *b*) was the first to rear the adults of these mining larvae and discovered them to be *Agromyza carbonaria* Zett. Actually he obtained the adults by rearing out pupae which he had found at the base of some alder trees. Then as *A. carbonaria* considerably exceeded in size all the other species of *Agromyza*, except *A. lappa* whose larval habits were already known, Nielsen came to the conclusion that *A. carbonaria* was the adult of the larvae to be found in alder, hazel, birch, willow, mountain ash, *Pyrus* and *Prunus*. It is exceedingly doubtful if it is the same species whose larvae attack such a wide range of trees. Nielsen described its life history and also summarised the literature on the subject. The next species to be reared and so definitely associated with fleck marks was *Agromyza pruinosa* Coq. which Greene (1914) ascertained was the cambium miner of river birch in America. Another species was reared from *Prunus avium* and *P. domestica* by Grossenbacher (1915) who

¹ Dr Hendel has proposed the name *Dizygomyza barnesi* sp.n. for this fly.

² The damage noted were discoloured spots in the wood and are variously described as “fleck marks,” “Markflecke,” “pith-ray flecks,” “pith flecks” and “medullary spots.”

described it as *Agromyza pruni* Gross. This was redescribed by Malloch (1915). Two further species were discovered in America and described by Greene (1917) as *Agromyza aceris* Greene and *Agromyza amelanchieris* Greene. In Europe a further species was reared by de Meijere (1925) from *Salix* and described by Hendel (1931) as *Dizygomyza cambii* Hendel.

The species discussed in this paper may therefore be considered as the third European species to be investigated, while three other species have received attention in America.

Mr Collin has suggested to me that the cambium miners to be found in other trees (besides willow) such as birch belong to different species.

III. MORPHOLOGY.

(a) *The egg.*

The egg (Text-fig. 1) is cylindrical with the posterior extremity broadly rounded whilst the anterior end is more pointed. It is opaque white and measures about 0.85 mm. in length and just over 0.2 mm. at its widest point. The micropyle at the cephalic pole is very obvious and is enshrouded by a supposedly gelatinous covering which protrudes beyond the anterior end of the egg in a comb-like structure. The chorion has a reticulated surface as indicated in the figure.

(b) *The larva.*

General and cuticular processes. The general character of the larva is shown in Text-fig. 2. It is opaque white, long and narrow, reaching a length, when full grown, of 18 mm. with a width of 1 mm. When first exposed in its burrow by peeling off the skin of the willow rod, the larva appears flattened, dorso-ventrally compressed, and greatly elongated, but it immediately assumes its correct tubular shape and contracts in length.

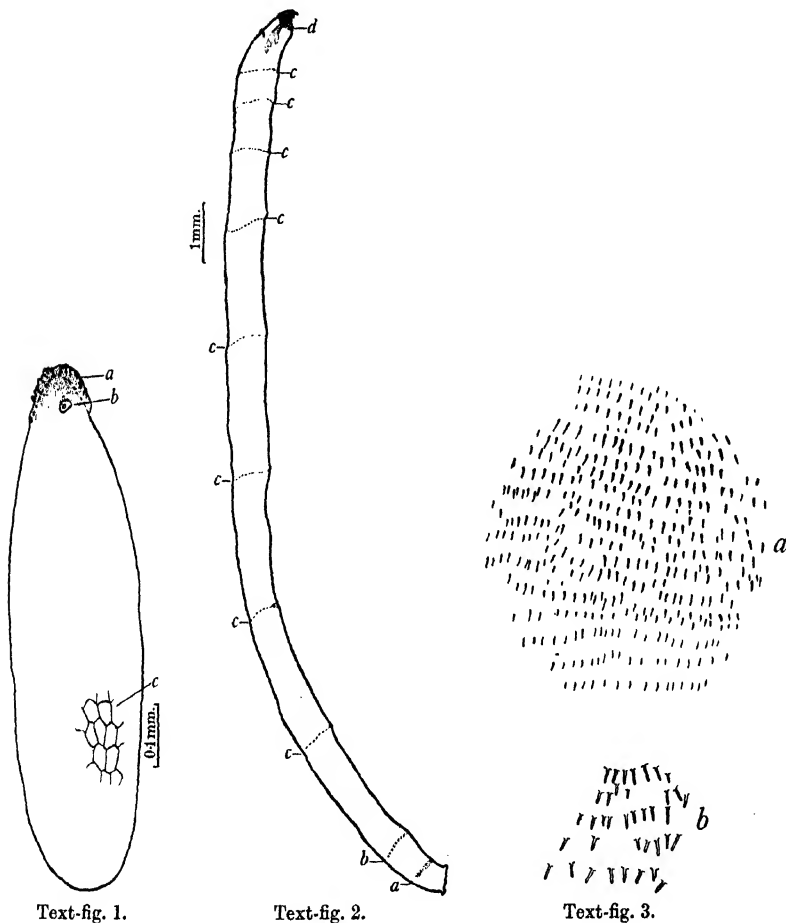
There are two types of cuticular processes, (a) those on the prothoracic segment, and (b) those on the segmental lines. Both sets of processes aid the forward movement of the larva in its burrow or mine and retards its movement backwards.

(a) On the prothoracic segment there are numerous microscopic chitinous finger-shaped processes (Text-fig. 3), which are directed backwards. They are arranged in about thirteen roughly parallel rows which form a collar at the anterior end of the segment.

(b) The second type is illustrated in Text-fig. 4. They are brown, chitinous and scale-like in appearance, directed posteriorly and arranged in rows. Starting with the anal segment, on each side at about one-eighth

502 *A Cambium Miner of Basket Willows (Agromyzidae)*

of the segment anterior to the posterior spiracles are 4-5 rows of such structures (a), then at about three-eighths of the segment are two such



Text-fig. 1. The egg. a, gelatinous covering; b, micropyle; c, indication of surface reticulation. $\times 83$.

Text-fig. 2. The larva. a, b, c, rows of scale-like cuticular processes; d, collar of finger-shaped cuticular processes. $\times 8$.

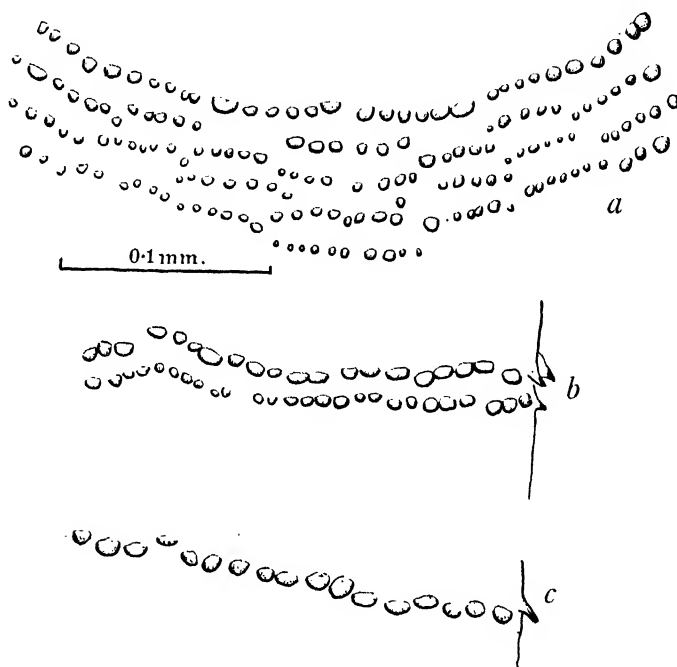
Text-fig. 3. Portions of the collar of finger-shaped cuticular processes. a $\times 385$, b $\times 600$.

rows (b) which extend completely round the body except for a small broken space ventrally. Anterior to these, corresponding roughly to the intersegmental spaces, are eight single rows (c) of similar processes.

These single rows appear to encircle the body. For the position of the rows see Text-fig. 2 (*a*, *b* and *c* correspondingly).

Greene uses the most posterior rows of these cuticular processes to separate the larvae of *Agromyza aceris* Greene from those of *A. amelan-chieris* Greene.

The cephalo-pharyngeal skeleton (Text-fig. 5). The mouth-hook (*m.h.*) is single and has a large median apical tooth (*a*). On each side there are



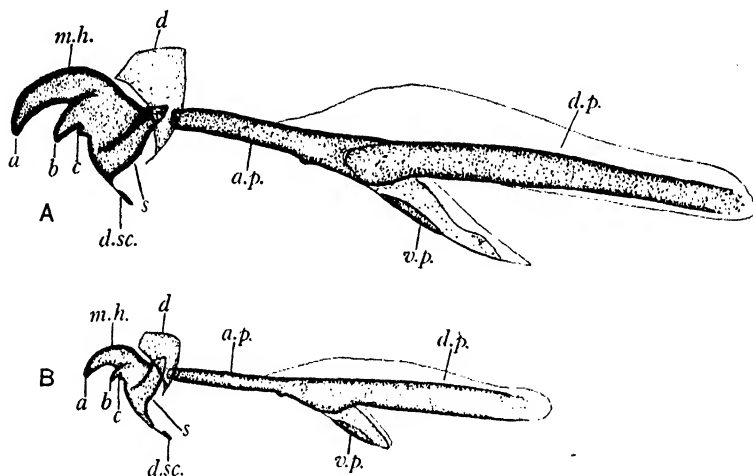
Text-fig. 4. Rows of scale-like cuticular processes. *a*, posterior set of rows; *b*, double row on the anal segment; *c*, single row on anterior segments of larva. $\times 275$.

two unequal smaller teeth (*b*, *c*). This single mouth-hook and large median apical tooth must be the result of a fusion of the two usual mouth-hooks. Hendel (1931, p. 6) states that two mouth-hooks are always present and figures those of *Dizygomyza postica* Meig. in his Text-fig. 14. The posterior portion (*s*) of the mouth-hook is separated from the anterior part, which bears the teeth, and forms a shield on either side of it, being joined to it at the base only. A slightly chitinated piece (*d*) is situated by the side of the mouth-hook and the anterior process of the pharyngeal sclerite. Below the ventral surface of the mouth-hook there

504 *A Cambium Miner of Basket Willows (Agromyzidae)*

is a small elongate piece which is probably the dentate sclerite (*d.sc.*). The antennal organ is situated immediately above the median apical tooth of the mouth-hook. In the last instar the anterior process of the pharyngeal sclerite (*a.p.*) appears to be separate from the dorsal process (*d.p.*) and the ventral process (*v.p.*), whereas in the penultimate instar there is complete fusion.

The spiracles. In the penultimate and ultimate instar the larva is amphipneustic. The anterior spiracles are situated on the dorsum of the prothoracic segment just posterior to the collar of cuticular processes.

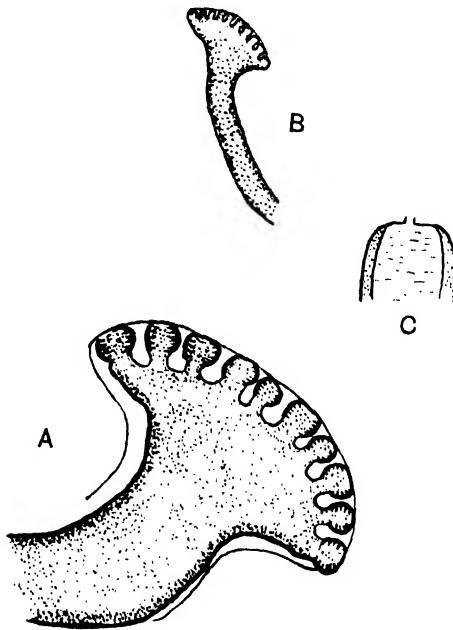


Text-fig. 5. Cephalo-pharyngeal skeleton. A, last instar larva; B, penultimate instar. $\times 275$. *a*, apical tooth; *b*, *c*, lateral teeth of mouth-hook; *a.p.* anterior process of pharyngeal sclerite; *d*, chitinated piece; *d.sc.* dentate sclerite; *d.p.* dorsal process of pharyngeal sclerite; *m.h.* mouth-hook; *v.p.* ventral process of pharyngeal sclerite; *s*, posterior portion of mouth-hook.

In lateral view they are rosette-shaped with a varying number (8–12) of cup-shaped bulbs. The openings are minute pores situated on a slightly raised projection (Text-fig. 6). Viewed from above, the spiracles are long and narrow showing one opening on each bulb. In colour they are pale yellow and are slightly raised above the surface.

The question of the exact number of cups or bulbs is interesting. In the penultimate instar (of which unfortunately only one specimen was obtained by actually seeing the moult), there are 10 such cups on one side while there appear to be 11 on the other. In the last instar of which many specimens have been examined the number varies most definitely. In 10 specimens the variation is as follows: 9 and 10 (the spiracle which

has 9 cups has the 5th and 6th cups fused), 8 and 11, 10 and 11, 10 and 10 (one spiracle has the 8th and 9th cups fused), 9 and 10, 9 and 10, 10 and 11, 10 and 12, 10 and 11, and 9 and 11. It has not been possible to see whether the lower number of cups is constantly situated on the spiracle of one side or the other. This variability in number throws doubt upon their use as diagnostic characters of the different instars, especially as in the one specimen of the penultimate instar the number appears to be the same as in the last instar. Steel (1931), working on the frit-fly,



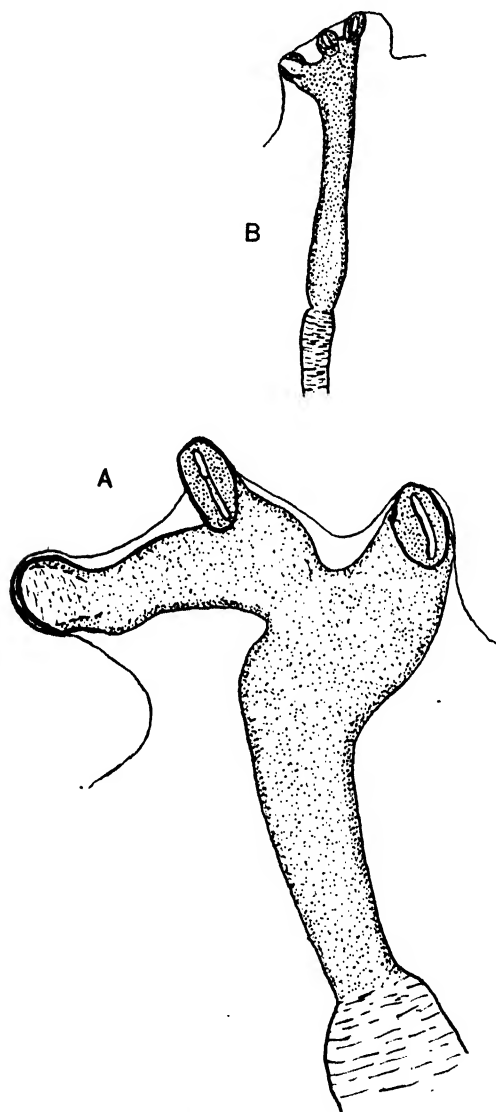
Text-fig. 6. Anterior spiracle of: A, last instar larva; B, penultimate instar larva; $\times 578$.
C, diagram of single bulb of spiracle showing aperture.

found that the normal number of digitate processes of the third instar larva was 6, although in some cases there were only 5 as in the second instar larva.

The posterior spiracles (Text-fig. 7) are pale yellow and have three chitinised plates each with a long narrow slit. The three plates are asymmetrical, one being borne on a longer arm than the other two, while the stigmatic chamber leads off from these latter two plates. In both the penultimate and last instar the number of the plates is constant and is three. This is in direct contrast to the observations of Nielsen (1906 *a*),

506 *A Cambium Miner of Basket Willows (Agromyzidae)*

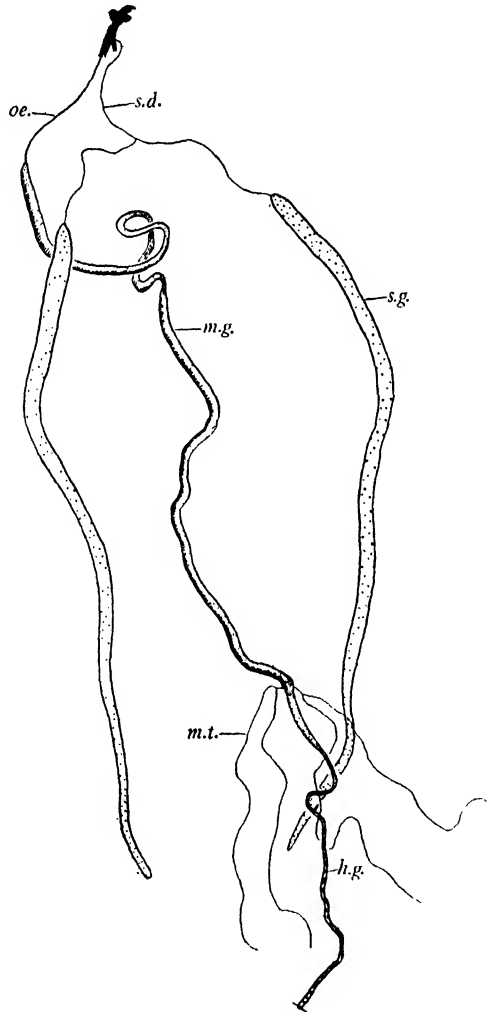
who figures the posterior spiracle of the first instar as having one plate, the second instar two and the third instar three plates.



Text-fig. 7. Posterior spiracle of: A, last instar larva; B, penultimate instar larva. $\times 578$.

The digestive system (Text-fig. 8). The alimentary canal is divided into three easily recognised regions: (a) the fore-intestine, (b) the mid-intestine and (c) the hind-intestine.

The oesophagus is a single narrow tube being only about twice as large in diameter as the salivary duct. The stomach or mid-gut is a



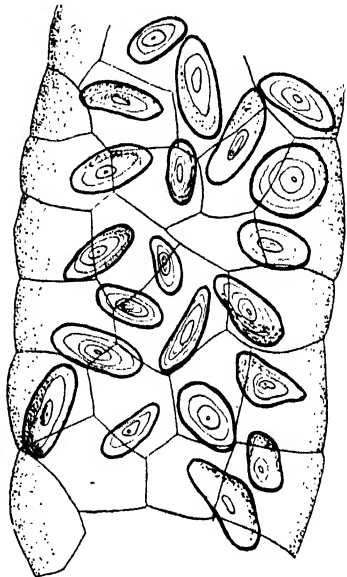
Text-fig. 8. Digestive system of larva. *h.g.* hindgut; *m.g.* midgut; *m.t.* Malpighian tubules; *oe.* oesophagus; *s.d.* salivary duct; *s.g.* salivary gland. $\times 8$.

long wide tube which is slightly coiled. The hind-intestine starts at the insertion of the Malpighian tubes, but at this point and for some little distance it is of the same size as the mid-gut. Hence the small intestine is not very well differentiated from the colon. More posterior lies the

colon which is of smaller diameter than the small intestine. The rectum is poorly defined.

The excretory organs and fat-body. I. The Malpighian tubes (Text-fig. 8). There are two pairs of Malpighian tubes, each pair becoming fused just before entering the alimentary canal.

II. The fat-body (Text-fig. 9). The fat-body is very conspicuous on account of the calcospherites which it contains. The latter are exceedingly numerous and must number several hundreds at least. Keilin (1921) figures the larva of an *Agromyza* species containing 120 such bodies and the writer has a specimen from lettuce leaves containing about 60-70. The calcospherites consist of calcium carbonate, and this storage of a waste product is typical of a large number of Diptera which are either parasitic, phytophagous or living in putrefying substances. A good account of this phenomenon is given in the above-mentioned paper by Keilin. One of his conclusions may be quoted with advantage. "During the first days of metamorphosis the calcium carbonate dissolves in the perivisceral fluid (haemolymph or blood of insects) and then passes through the newly formed pupal cuticle into the ecdysial fluid. When the latter is absorbed, the calcium carbonate remains as a deposit upon the internal surface of the puparium." It has been observed in the species under consideration that the newly formed pupa contains the calcospherites but that older pupae do not. Also that the empty puparia are lined with a whitish powdery substance which effervesces on coming in contact with dilute acid. These observations endorse Keilin's conclusion of ecdysial elimination as quoted above.



Text-fig. 9. Portion of fat-body, showing calcospherites. $\times 115$.

The glands or organs of secretion. Labial glands (Text-fig. 8). The labial glands or salivary glands are very prominent and large. They are of normal structure, being paired and their ducts uniting to form a common duct. The cells of the glands are large and would be ideal for histological study.

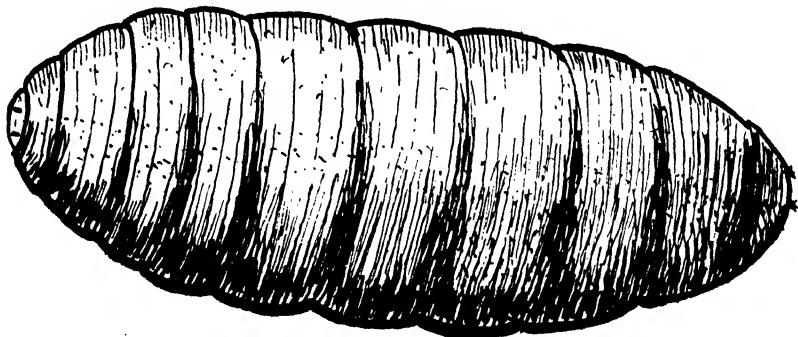
(c) *The pupa.*

The pupa (Text-fig. 10) is of normal coarctate shape, the puparium being cylindrical, dark reddish brown and about 4-5 mm. in length. Both the anterior and posterior spiracles are inconspicuous.

(d) *The adult, Dizygomyza barnesi sp.n. Hendel.*

The adult (Plate XXIX, figs. 1, 2 and 3) has been described for me by Dr Hendel as follows:

"Dr Barnes' fly (*Dizygomyza barnesi* sp.n.) may be distinguished in the following manner. Width of front almost one and half that of eye, as long as broad; width of each orbit (behind) about one-sixth that of frontal stripe; 4-5 orbital bristles, the hindmost bend backwards; parafacials broad, *not smaller below than above*; height of cheeks equal to about one-fourth the eye height; eyes scantily haired. Mesonotum with



Text-fig. 10. Pupa. $\times 25$.

3 + 1 dorsocentral bristles; the third appreciably nearer to the suture than the second *dc*; the praescutellar pair between the first pair of *dc*, not before it as in *carbonaria*; 8-9 irregular rows of acrostichal hairs. No praesutural intra-alar bristle as in *carbonaria*. One or two postdorsal bristles on mid tibiae, while *carbonaria* and *cambii* have no distinguishable bristles there. The colour of the whole fly is black; frontal stripe opaque, orbits and ocellar triangle slightly glossy; mesonotum, scutellum and abdomen with greyish pollinosity, only slightly shining. Sixth abdominal segment elongated; base of ovipositor shorter than the 6th segment, almost cylindrical, transversely wrinkled. In all other respects the fly resembles *Dizygom. carbonaria* (Zett.) Hend. Length $3\frac{1}{2}$ -4 mm."

Dr Hendel possesses a male and a female which I sent him, while I am in possession of another male and female which were also examined by him. These specimens were obtained from material collected in Hertfordshire in 1931.

IV. DISTRIBUTION.

This insect is common in basket willow beds at Mawdesley near Ormskirk, Lancashire, and it occurs in willow beds at Batford near Harpenden, Hertfordshire. Specimens have also been received from Beccles, Suffolk (through Ministry of Agriculture) and from near Selby, Yorkshire.

Search has been made for its occurrence by willow growers in Devon, Essex, Leicestershire, Lincolnshire and Somerset, but without success.

Inquiries have been sent to growers in other districts of England, Ireland, Scotland and the Isle of Man, but without result.

It seems probable, however, owing to its wide distribution, that it will be reported from other districts when it becomes better known.

V. BIONOMICS.

(a) Emergence, mating and oviposition.

Emergence of the adults takes place at Harpenden from about May 21st until the end of June. The flies remain alive for about a week in captivity.

Mating has only been observed once. No signs were seen of any courtship and actual coition lasted from 8.30 a.m. until 1.45 p.m. (standard time). Egg-laying started in this case at 2.30 p.m. the same day.

When about to oviposit the female walks up and down a willow stem and usually chooses a point about 10 in. to a foot from the ground on a young shoot in its first year of growth. Having selected the spot, the fly bunches up its abdomen and puts its ovipositor at right angles to the plant epidermis; then it starts boring into the stem. This proceeds with a steady pushing motion and the fly moves backwards slightly as the ovipositor sinks into the tissue, until the abdomen is again horizontal to the surface of the stem. Then it remains perfectly still for an instant while the egg passes down the ovipositor into the stem. The ovipositor is withdrawn and the insect generally flies away to another shoot before repeating the process. A single egg is laid in each puncture. A small hole in the epidermis is all that is visible, the egg being placed right in the tissue with only the anterior end penetrating into the inner layers of the epidermis.

(b) Damage.

The damage done is caused by the larvae tunnelling up and down the stem in the subcortical layer or cambium. The larvae confine their activities to the basal portion of the rods, the stub and the roots. Thus

when the rods are grown for sets the most valuable portion is useless. Similarly for basket work the thick end of the rods has to be thrown away. As plant growth continues these tunnels become covered by new tissue. The result is that the rod is weakened throughout. This becomes of greater importance when the rods are grown for stake rods, *i.e.* for 2 years or more. Finally, if the willow were to be grown as a tree for wood it would be useless owing to the so-called fleck marks which are scattered throughout any cross-section. In Plate XXX these various types of damage are illustrated. Fig. 1 is a slightly diagonal section of a 4- or 5-year-old set of *Salix viminalis* and shows the fleck marks. Fig. 2 is the cross-section of a 2-year-old rod of Long Skin variety of *S. viminalis*. The rod was tunnelled slightly in the late summer or autumn growth of the first year, very badly in the early growth of the second year and slightly again in the second autumn growth. Fig. 4 is the same rod in longitudinal view, when peeled, and shows a tunnel exposed. The two smaller white objects are the cocoons of the gall midge inquiline which is to be described later in this paper. The larger white object in the burrow is the larva of the fly.

Besides the damage done by the actual tunnelling, the tunnels themselves are points exposed to bacterial and insect attack. This frequently takes place. In such cases rot quickly sets in and the stems soon have an appearance very similar to normal attack by black canker. Plate XXX, fig. 5, shows the external appearance of a rod primarily attacked by the fly and secondarily infected with rot. Fig. 6 on the same plate shows the appearance of the rod when partially peeled. In both these figures as well as in figs. 2 and 4 (Plate XXX) cavities can be seen caused by the secondary infection. It is probable that black canker itself may follow the primary fly damage.

The easiest way of detecting the presence of this cambium miner is to look for cankerous rods and then cut the rod in order to see whether the typical fleck marks as seen in Plate XXX, figs. 1, 2 and 3(1) are present or not. Alternatively one can peel the rods at their bases in order to expose the tunnels, but this is only possible at those seasons of the year when the tunnels are near the surface. Except in cases of secondary canker and very severe attack, when there may be several larvae burrowing in a single rod, there is very little change in the external appearance of the growing rods. Sometimes, however, there is a slight discoloration and sinking inwards of the skin immediately over the burrow. The damage is most noticeable in August and September and when the rods are peeled.

(c) Host plants.

The most usual species of basket willow to suffer attack is *Salix viminalis*, of which the varieties Dark Long Skin, Greenskin and, to a less extent, Continental are prone to damage. In addition, larvae and tunnels have been found in Lancashire in commercial beds on the following varieties and species: Pomeranian (*S. triandra*), Dicky Meadow (*S. purpurea*), Harrison (*S. viminalis* × *purpurea*) and Black Top (*S. triandra* × *viminalis*). In Yorkshire it has been found on *S. viminalis* chiefly and also on *S. purpurea*.

In greenhouse experiments at Rothamsted it has been successfully reared on Long Skin (*S. viminalis*). Attempts have also been made to rear it on Dicky Meadow, Black Maul (*S. triandra*) and Cricket Bat Willow (*S. caerulea*). In the case of the last-named species, the adults were observed laying their eggs very readily, but the larvae died before reaching maturity. This was almost certainly due to failure on the part of the writer to keep the plants in good condition during the hot weather of July, 1932. The females used in the experiments on Dicky Meadow and Black Maul failed to lay eggs; this, however, was probably due to the fact that only old females were available. Field records however have shown that Dicky Meadow (*S. purpurea*) and at least one variety of *S. triandra*, viz. Pomeranian, are liable to attack.

In view of the peculiar interest of its being able to oviposit and live on Cricket Bat Willow, search has been made where possible for its occurrence on this species in the field. But so far it has not been found for certain although the writer has been told of one case where fleck marks, which might have been due to this insect, were supposed to have been seen.

Apparently this fly prefers soft wooded varieties to hard ones.

(d) Life cycle.

The egg stage lasts from about a week to a fortnight. As soon as the larva hatches, it starts mining downwards towards the stub, remaining throughout its life in the cambium tissue. Its passage can be traced by the fine tunnel it leaves immediately below the skin. Its progress is irregular but there is a downward tendency for the greater part of June and July. Cast skins can be found in the tunnels which appear to sink inwards as fresh plant growth takes place and covers the old passage. The tunnelling has been found right down to the tips of the smaller roots but never more than about 3½ ft. up the rods. Towards the end of July the larvae change their direction and start working upwards. When ready to pupate the larva makes a slit in the outer skin of the rod (see

Plate XXX, fig. 3) and, coming out, gets to the soil. It has not been observed exactly how this is accomplished. It appears probable that the larvae either crawl down the outside of the rods in damp weather or, if it is too dry for this, remain on the rod near the exit holes until they have become much shorter in length preparatory to pupation and then drop to the ground. The exit holes may be found up to about 3 ft. up the rods but frequently occur in the bottom foot. They are quite characteristic. It is possible that some of the larvae make exit holes on the stubs but none have been found in this position or on the roots. These slits or exit holes have been found as early as July 21st, while larvae have been found as late as September 16th. Usually, however, most of the larvae come out of the rods during August.

Pupation takes place in the top few inches of soil around the stubs and the puparia remain here throughout the winter and spring. They have been found as early as July 28th on occasion.

Emergence of the adults occurs the following May and June. There is only one brood a year.

VI. PARASITES.

The following Braconid parasites have been reared: *Symphia ringens* Halid. and *Symphia hians* Nees (Lancashire and Hertfordshire). Both these parasites are illustrated in Plate XXIX, figs. 4 and 5. A further Braconid, *Apanteles fulvipes* Halid., is provisionally associated as a possible parasite of the *Dizygomyza*.

It is interesting to note that Greene (1914) records *Sympha agromyzae* Rohwer as a parasite of *Agromyza pruinosa* Coq.

Parasitism by the *Symphia* species appears to be quite high. Adequate numbers have not been reared to be certain of this, but from Lancashire material in 1931 three flies and one *Symphia* were reared; in 1932 from material collected in the same locality six *Symphia* only were bred. From Hertfordshire material, sample A gave seven flies and four parasites and sample B five flies and three parasites, both in 1932.

However, the parasitism is not sufficient to prevent the flies being numerous enough to cause very prevalent damage in these two localities. Sometimes hardly a rod of Dark Longskin is free from attack, in other years 50 per cent. of the rods are tunnelled.

VII. INQUILINE GALL MIDGE, *PROFELTIELLA DIZYGOMYZAE* SP.N.

The larvae of this gall midge live as inquilines in the burrows of the cambium miner. The adult midge is very distinctive in coloration, the wings being bright yellow mottled with black, the legs banded with yellow and black and, in the case of the female, the abdomen bright red. It is also of moderate size.

Two species in this genus have previously been described, firstly *P. ranunculi* (Kieffer) which was originally placed in the genus *Lestodiplosis* (Kieffer, 1909). Later Kieffer (1912) raised the genus *Profeltiella* for this species. The larvae of *P. ranunculi* were stated to feed on the larvae of another gall midge, *Geodiplosis ranunculi* Kieffer, which live on the roots of *Ranunculus acer* L. in Germany. The larvae of both these species have been recorded from Northumberland by Bagnall and Harrison (1922). The second species is *P. orientalis* Felt reared in association with the gall midge *Kamptodiplosis reducta* Felt from leaf galls on *Siphonodon celastrineus* Griff. in the Philippine Islands. Felt (1918) tentatively placed it in this genus.

The species under consideration can be easily distinguished from either of the above species by the colour of its legs which in *P. ranunculi* are black and white and in the case of *P. orientalis* yellow to straw. There are various other differences. It has been decided to describe this species under the name *P. dizygomyzae* sp.n. on account of its larval habit.

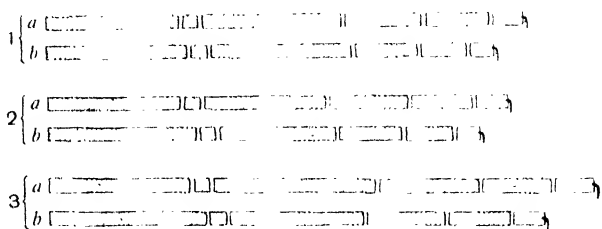
(a) *Description.*

Male. Length about 2 mm. Antennae: 2 + 12, proximal basal segment small, distal basal segment roundly quadrate; 1st and 2nd flagellar segments fused; basal enlargement on two proximal flagellar segments globular, on more distal segments subglobular, each such enlargement with ring of stout setae and a single whorl of regular circumfila; distal enlargement elongated, each bearing two whorls of regular circumfila and one ring of stout setae just proximal to distal circumfila; stem of 3rd flagellar segment about $1\frac{3}{4}$ -2 times as long as broad, neck about $2\frac{3}{4}$ -3 times as long as broad; stem of distal segment about $4\frac{1}{2}$ times as long as broad, distal enlargement about 4 times as long as broad. Palpi: each segment with short setae; proximal segment rectangular, the length half as long again as width; 2nd segment just over twice as long as first and slightly over 3 times as long as broad; 3rd segment about 3 times as long as and slightly narrower than first, about $4\frac{1}{2}$ times as long as broad; distal segment nearly 4 times as long as and slightly

narrower than first, about 7 times as long as broad. Wings: mottled yellow suffused with black, 3rd vein reaching margin just beyond tip of wing, costa interrupted at this point. Legs: distinctive, yellow and black, caused by the different colours of the hairs, proportions of black and yellow on tibia and tarsus of fore-, mid- and hind-legs as in female; claws curved at right angles, those of fore-legs bifid, those of others simple, empodium small. Genitalia: basal clasp segment stout with slight lobe; distal clasp segment moderately narrow; dorsal lamella deeply bilobed, each lobe rounded; ventral lamella about as long as or slightly longer than dorsal lamella, broad and roundly emarginate.

Co-types, Cecid. 1556, 1558, 1618, 1619, 1914-16 inclusive.

Female. Length about $2\frac{1}{2}$ mm. Antennae: 2 + 12, basal segments as in male; 1st and 2nd flagellar segments fused, 3rd flagellar segment about 3 times as long as broad, neck slightly longer than broad; distal



Text-fig. 11. Diagram of legs of: *a*, *Profeltiella dizygomyzae* sp.n.; *b*, *Profeltiella respicoloris* sp.n. to show different proportions of black and yellow coloration. 1, fore-leg; 2, mid-leg; 3, hind-leg.

segment about $3\frac{1}{2}$ times as long as broad, distal enlargement about 3 times as long as broad, circumfila applied. Palpi: about as in male. Wings: deeper coloration than in male. Legs: black and yellow, proportions of black and yellow on tibia and tarsal segments of fore-, mid- and hind-legs as in Text-fig. 11 *a*. Ovipositor: lamelliform, very extensile, nearly as long as abdomen. Otherwise about as in male.

Co-types Cecid. 1557, 1909-13 inclusive.

Pupae, Cecid. 1669.

Larvae; gregarious, red, recognisable as *Profeltiella* sp. by the anal segment (Text-fig. 12).

Cecid. 1501-1503 inclusive.

Habitat. Larvae live in mines of *Dizygomyza barnesi* Hendel on *Salix* spp.

Another species of *Profeltiella*, of which a single female has been reared, is very similar to *P. dizygomyzae* but must be considered a distinct

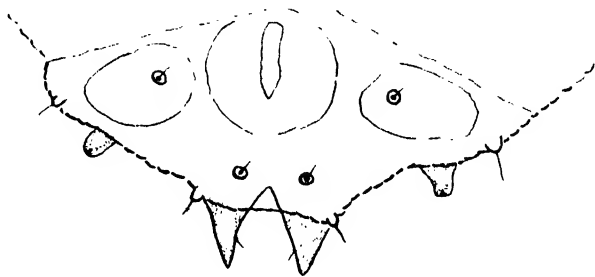
516 *A Cambium Miner of Basket Willows (Agromyzidae)*

species. It is proposed to name this species *P. vespicoloris* sp.n. and it can be described as follows:

Female. Very similar to female of *P. dizygomyzae*. May be separated from this species by the proportions of black and yellow on tibia and tarsal segments of fore-, mid- and hind-legs as shown in Text-fig. 11 *b*, in particular the 2nd tarsal segments are more yellow than in *P. dizygomyzae*. Palpi: very similar proportions to those of above species.

Type, Cecid. 1473.

Habitat. Single example reared from pot of soil in which *Dasyneura arabis* Barnes was being reared on *Arabis albid*a at Rothamsted, June, 1930, 3 months previous to the first finding of *P. dizygomyzae* larvae.



Text-fig. 12. Anal segment of larva of *P. dizygomyzae* sp.n. (diagrammatic).

(*b*) *Distribution.*

This midge seems to be widely distributed wherever the cambium miner occurs and is definitely associated with it. It has been reared from material collected in Lancashire and Hertfordshire, while larvae were found in the material sent from Suffolk.

(*c*) *Bionomics.*

The larvae live gregariously in the burrows of *D. barnesi* and can be found in large numbers in the larger cavities caused by rot setting in after the primary fly damage. Such cavities are doubtless partially due to the presence of these larvae in a burrow as well as to rot. The larvae are bright salmon pink in colour and may be recognised by the anal processes. When they are full grown, in August and September, they separate to some extent and spin white cocoons, still remaining in the burrows and cavities. Some viable larvae and pupae can, however, be found in the soil around the stubs, probably having fallen accidentally out of the rods. Pupation takes place in the spring, 8 days or so before emergence, although the cocoons are spun in the autumn. Plate XXX,

fig. 4, shows two cocoons, one at the terminal end of a tunnel and the other in a cavity.

Just before emergence of the adult the pupa wriggles its way along the burrow or from the cavity to the surface by means of the stiff spines on its dorsal surface. Normally emergence takes place in July, but pupae kept indoors in the laboratory from September onwards emerged successfully but spasmodically between January 26th and May 27th.

There is only one brood a year. So far this midge has only been found on *Salix viminalis*.

(d) *Parasites.*

The following Scelionid parasite has been reared from *Profeltiella dizygomyzae* collected in Lancashire: *Ectadius craterus* Walk. The parasitism figures vary considerably from year to year; in 1931 about equal numbers of midge and parasite were reared, but in 1932 36 parasites were reared to two midges. The parasite can be made to emerge earlier than usual by keeping it indoors in the winter, thus resembling its host.

VIII. SUMMARY.

1. A brief résumé is given of the information available concerning Dipterous cambium miners (Agromyzidae).

2. The morphology of the egg, larva, pupa and adult of *Dizygomyza barnesi* sp.n. Hendel is described.

3. The bionomics of this fly receive detailed attention. There is only one brood a year and the larvae mine in the cambium of different species of *Salix*. Pupation takes place in the soil. While *S. viminalis* is attacked most commonly, *S. triandra*, *S. purpurea*, *S. viminalis* × *purpurea* and *S. triandra* × *viminalis* occasionally suffer. In addition the larvae can live on *S. caerulea*. The damage caused by the larvae is considerable. The Braconids, *Symphia ringens* Halid. and *S. hians* Nees, are recorded as primary parasites.

4. Two gall midges are described, the larvae of one living as inquiline in the burrows of the *Dizygomyza* larvae. The bionomics of this species are described.

REFERENCES.

The titles of those papers referring solely to the classification and distribution of gall midges are omitted, only the journal reference being given.

- BAGNALI, R. S. and HARRISON, J. W. H. (1922). *Ent. Rec.* XXXIV, 149-50.
- BROWN, H. P. (1913). Pith-ray flecks in wood. *Forest Ser. U.S. Dept. Agric. Circ.* 215, 5-15.
- FEIT, E. P. (1918). *Philippine Journ. Sci.* XIII, 292-3.
- GREENE, C. T. (1914). The cambium miner in river birch. *Journ. Agric. Res.* I, 471-4.
- (1917). Two new cambium miners (Diptera). *Ibid.* x, 313-17.
- GROSSENBACHER, J. G. (1910). Medullary spots: a contribution to the life history of some cambium miners. *N. Y. Agric. Exp. Sta. Tech. Bull.* No. 15, 49-65.
- (1915). Medullary spots and their cause. *Bull. Torrey Bot. Club*, XLII, 227-39.
- HENDEL, F. (1931). Agromyzidae in Lindner's *Die Fliegen der palaearktischen Region*, No. 59, 1-26.
- KEILIN, D. (1921). On the calcium carbonate and the calcospherites in the Malpighian tubes and the fat body of Dipterous larvae and the ecdysial elimination of these products of excretion. *Quart. Journ. Micro. Sci.* LXV, 611-25.
- KIEFFER, J. J. (1909). *Bull. Soc. Hist. nat. Metz*, XXVI, 24-5.
- (1912). *Neue Gallmücken-Gattung*, Bitsche and *Marcellia*, XI, 10-11.
- KIENITZ, M. (1883). Die Entstehung der Markflecke. *Bot. Centrbl.* XIV, 21-26, 56-61.
- MALLOCH, J. R. (1915). Some additional records of Chironomidae for Illinois and notes on other Illinois Diptera. *Bull. Ill. St. Lab. Nat. Hist.* XI, art. 4, 349-50.
- DE MELIERE, J. C. H. (1925). Die Larven der Agromyzinen. *Tijdsch. v. Entom. Neder. Entom. Vereen.* LXVIII, 259-61.
- NIELSEN, J. C. (1906 a). Zoologische Studien über die Markflecke. *Zool. Jahrb. Abt. System. Geogr. u. Biol. Tiere*, XXIII, 725-38.
- (1906 b). Über die Entwicklung von *Agromyza carbonaria* Zett., der Urheber der "Markflecke." *Zool. Anz.* XXIX, 221-2.
- RECORD, S. J. (1911). Pith flecks or medullary spots in wood. *For. Quart.* IX, 244-51.
- STEEL, A. (1931). On the structure of the immature stages of the frit fly (*Oscinella frit* Linn.). *Ann. Appl. Biol.* XVIII, 352-69.

EXPLANATION OF PLATES XXIX AND XXX.

PLATE XXIX.

- Fig. 1. A cambium miner of basket willows *Dizygomyza barnesi* sp.n. Hendel. Adult ♀, with outstretched wings. × 8.5.
- Fig. 2. The same, with wings in resting position. × 10.
- Fig. 3. The same, to show wing venation. × 20.
- Fig. 4. *Symphia ringens* Halid., parasite of *Dizygomyza barnesi*. × 10.
- Fig. 5. *Symphia hians* Nees, parasite of *Dizygomyza barnesi*. × 10.

PLATE XXX.

- Fig. 1. Section of 4-5-year-old *Salix viminalis*, showing fleck marks due to the tunnelling of the larvae of the *Dizygomyza*. Slightly diagonal. Natural size.
- Fig. 2. Cross-section of 2-year-old rod of Long Skin variety of *S. viminalis*, showing fleck marks and cankerous rot setting in. Slightly reduced.



Fig. 1.



Fig. 2

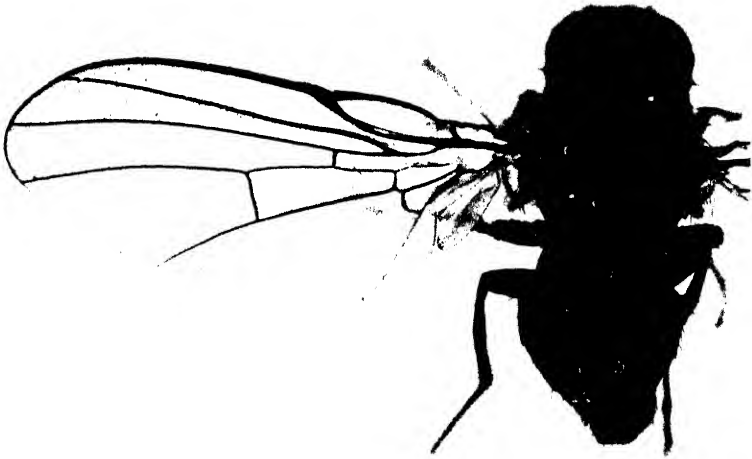


Fig. 3.

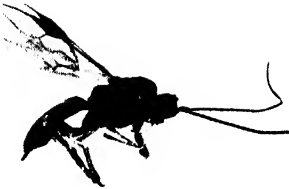


Fig. 4.



Fig. 5.

BARNES.—A CAMBIUM MINER OF BASKET WILLOWS (*AGROMYZIDAE*) AND ITS INQUILINE GALL MIDGE (*CECIDOMYIDAE*) (pp. 498-519).



Fig. 1.



Fig. 2.

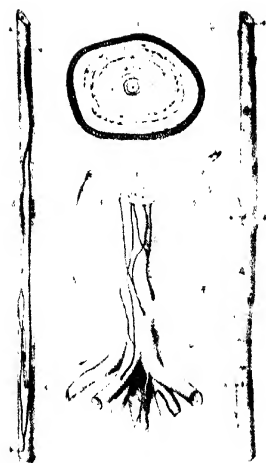


Fig. 3.



Fig. 4.



Fig. 5.



Fig. 6.

- Fig. 3. Reproduction of plate by Kienitz (1883). 1, cross-section of *Betula pubescens*, showing fleck marks. 2, peeled rod of *Salix rubra* Huds., showing tunnelling of the larva. 3, lower part of *Sorbus aucuparia*, showing fleck marks and tunnelling. 4, unpeeled rod of *S. rubra*, showing larva and exit hole. 5, dipterous larva. 6, head of larva.
- Fig. 4. Base of peeled Long Skin (*S. viminalis*) rod, showing two cocoons of *Profeltella dizygomyzae* sp.n. in burrows of the *Dizygomyza*, a larva of which has been drawn in to indicate its appearance. Slightly enlarged.
- Fig. 5. Unpeeled rod of Long Skin (*S. viminalis*) showing cankerous appearance due to secondary attack by gall midge larvae and rot. Slightly reduced.
- Fig. 6. Same rod partially peeled, showing cavities in which the gall midge larvae congregate. Slightly reduced.

(Received January 9th, 1933.)

STUDIES OF FLUCTUATIONS IN INSECT POPULATIONS

III. THE GALL MIDGE, *RHABDOPHAGA HETEROBIA* H.L.W., ON BLACK MAUL VARIETY OF *SALIX TRIANDRA* AT SYSTON, LEICESTERSHIRE, 1927-33

By H. F. BARNES, M.A., PH.D.

(Entomology Department, Rothamsted Experimental Station.)

(With four Figures in the Text.)

CONTENTS.

	PAGE
I. Introduction	165
II. Methods	166
III. Identification and biology	167
IV. The fauna of the galls	170
V. Size of population of <i>Rhabdophaga heterobia</i> and its parasites, 1928-33	170
VI. Relative parasitism of the midge:	
(i) Identification	173
(ii) Methods and results	173
VII. Emergence	176
VIII. Summary	180
IX. Acknowledgments	180
References	180

I. INTRODUCTION.

THIS is the third of a series of papers giving the results of an attempt to study fluctuations in insect populations as they occur in nature. The first study (1) was concerned with the wheat blossom midges on an experimental field (Broadbalk) of permanent wheat at Harpenden, Herts. The second (2) dealt with the infestation of wild meadow foxtail grass (*Alopecurus pratensis*) near Aberdeen by the gall midge *Dasynura alopecuri* (Reuter).

In the present contribution an account will be given of the "button top" midge or *Rhabdophaga heterobia* H.Lw., as it has occurred in a bed of commercially grown Black Maul (*Salix triandra*) basket willows near Syston, Leicestershire, during the past six years.

Mr A. Roebuck of the Midland Agricultural College has very kindly written a paper (12) to accompany mine, in which he gives a general account of the particular willow beds from which material for this study has been obtained. He has pointed out how the species studied has been carefully nursed so that it is always present in immense numbers, whereas there are other insects which have fared worse against flood and rain, etc. After dealing with the

other pests, he explains how a change in the method of working the beds would have probably prevented my study from being made.

The previous studies are resembled in so far that the degree of parasitism of the midge and the dates of emergence of the adult midges have been studied. But the varying extent of infestation of the crop has not been considered in any detail.

It should be pointed out that, whereas in the two previous studies each successive brood of the insect has been considered (the midges in question being normally single-brooded), in the present instance each over-wintering brood only has received attention. The intervening broods have not been studied, except under experimental conditions (see section III).

II. METHODS.

The method of sampling has been reduced as far as possible to a maximum of simplicity. Once a year, at the very end of October or beginning of November, a visit has been made to the Wanlip willow beds near Syston, Leicestershire. On November 1st, 1927, a single sample¹ of 500 "buttons" or galls caused by the larvae of *R. heterobia* was collected during the process of wandering to and fro over a single field in such a manner that the whole of the field was traversed. The same procedure was followed on October 31st, 1928. On October 30th, 1929, however, three separate samples of 500 galls were collected in the same manner, each sample being kept as a unit. On October 31st, 1930, 1500 galls were collected and subsequently mixed and divided into three samples of 500 galls. In 1931 (30. x. 31) and 1932 (1. xi. 32) this same procedure was used. In each year the samples have been brought back to Rothamsted Experimental Station and kept continuously in an outdoor insectary until the subsequent emergence of the midges and parasites was completed.

This method of sampling is open to the criticism that in years when the galls are abundant it is probable that owing to the human factor large-sized galls would preponderate in the samples, whereas in years when the galls are scarce in order to reach the required number it would be necessary to pick galls of all sizes. This is a point to be considered because it is possible that the smaller sized galls may contain a higher proportion of parasitised midge larvae than the large-sized ones. On the other hand, the particular field in which the samples have been taken is always very heavily infested, practically every terminal and lateral shoot bearing a button, and it has never been difficult to collect sufficient numbers of galls. This high infestation is doubtless primarily due to the fact that the bolts of rods from all over these particular willow beds are brought into this field in which the pits are situated. Here the bolts are peeled and naturally an accumulation of midge larvae occurs; the heaps of

¹ Part of this sample was used for other purposes, leaving 488 galls for use in this study.

peelings near the pits also tend to increase the numbers of larvae present. No attempt has so far been made to destroy all these larvae, consequently when the adult midges emerge the nearest suitable place for oviposition is this field. From observation it is possible on occasion to see a diminution in intensity of attack as one proceeds from the end of the field which contains the pits towards the other end. Usually, however, this decrease only becomes obvious in the next field and further away from the first one.

When collecting the galls two rules have been observed: (1) care has been taken to reject any gall that has had its centre eaten out by birds, and (2) no choice has been exerted as to size of gall picked. Several persons have helped the writer in collecting the galls at different times. I am indebted to my wife, Mr A. Roebuck, and Mr H. C. F. Newton in this connection.

The samples of galls have been kept over winter in a lamp glass standing on muslin sewn on to an iron ring which is placed over a Petri dish containing water. A similar muslin-covered iron ring is placed over the top of the lamp glass. The water in the Petri dish evaporates through the samples of galls and so ensures the requisite degree of humidity. Occasionally (once a week in May, June and July) the top of the sample is sprayed with water in addition. This affects the numbers of midges emerging from day to day and temporarily sends up the emergence numbers. However, when weekly emergences are considered the effect is not appreciable.

Finally, the numbers of the midges and their parasites which have emerged have been noted daily, each day ending arbitrarily at 7 p.m. standard time (8 p.m. summer time).

III. IDENTIFICATION AND BIOLOGY.

The "button top" midge, *Rhabdophaga heterobia* H.Lw., is the best known of the gall midges which attack basket willows. The larvae cause galls known as "buttons" on the terminal shoots of many commercial varieties of *Salix triandra*, but recent work (3, 4) has shown that they cannot live on *S. viminalis*, *S. purpurea*, *S. viminalis* \times *purpurea*, *S. viminalis* \times *triandra* (Black Top) and *S. alba* var. *vitellina*. Later work has shown in addition that this species cannot live on *S. alba*, *S. caerulea*, *S. fragilis*, *S. nigricans*, *S. americana* and *S. repens*. Besides living in the buttons, the larvae also inhabit the male catkins and lateral buds.

Other midges with which it may be confused are *Rhabdophaga rosaria* H.Lw. and an unidentified midge whose larvae live in the terminal shoots of *Salix viminalis*, causing a dying off of the apex. The galls of *Rhabdophaga rosaria* are frequently to be found on wild *Salix* spp. of the broad-leaved type, and less often have been reported in commercial osier beds. The only attack so far observed by the author on commercially grown willows was on Cricket Bat willows (*S. caerulea*) being grown for sets in Norfolk. The midge and the gall are much larger than in the case of *Rhabdophaga heterobia*. The host plants

of *R. rosaria*, however, have not yet been critically examined. Magerstein (5) has recently dealt with a midge he has identified as *R. rosaria* and figures the galls on *Salix viminalis*, but in all probability he has misidentified the midge or else got several species confused.

A popular illustrated account of the bionomics of *Rhabdophaga heterobia* has recently been published (6), so that there is no need to rewrite it here.

In the field there appear to be two main broods a year, the adults of the over-wintering generation being on the wing from the end of April until the beginning of July and those of the summer brood being on the wing from July until September. In reality there is a succession of overlapping broods throughout the summer months, individuals of the last brood or broods remaining in the galls as fully fed larvae throughout the winter, unless they are scattered to the ground by birds pecking at the "buttons" or galls. Such individuals are able to survive in the soil and come to maturity.

While breeding this midge in an unheated open greenhouse at Rothamsted it has been the rule to obtain three flights of midges each summer; thus under these conditions there are three broods a year.

The time taken from when a newly emerged and mated midge commences to oviposit until the appearance of her first progeny in the adult stage has been studied in the summer broods. The average length of this period for the first brood in 1929 was 41 days with a percentage standard error of ± 3.93 ; that for the first brood in 1930 was 40 days with a percentage standard error of ± 1.65 . The average length of the same period for the second brood in 1929 was 35 days with a percentage standard error of ± 3.73 ; that for the second brood in 1930 was 44 days with a percentage standard error of ± 1.83 . The minimum time in both these broods and years was 33 days and the maximum 48 days. In all thirty-four experiments were involved. In 1933, in two experiments only, the time taken for the first brood was 38 days.

If we compare this unit of time for the first brood of 1929 with that of the second brood in 1929, using "Student's" t method¹, we arrive at the value 2.65 for t , which is significant. In the same way this unit of time for the first brood in 1930 can be compared with that of the second brood in 1930; similarly a comparison of the first brood in 1929 and the first brood in 1930 can be made, also between the second brood in 1929 and the second brood in 1930. The values for t in such comparisons are set forth in Table I.

Table I. Values for t for comparison of the shortest duration of life cycle in the summer broods of *Rhabdophaga heterobia* in 1929 and 1930.

Comparison	t	$P=0.05$
First 1929 brood with second 1929	2.65	2.45
First 1929 brood with second 1930	2.14	2.16
First 1929 brood with first 1930	0.82	2.11
Second 1929 brood with second 1930	5.78	2.16
First 1930 brood with second 1930	4.60	2.06
First 1930 brood with second 1929	2.98	2.11

¹ I am indebted to Mr F. J. Richards for assistance in this connection.

The result is that we find that the first brood in 1929 was significantly slower in reaching maturity than the second in 1929 and significantly quicker than the second in 1930. On the other hand, the first brood in 1929 reached maturity in about the same time as the first brood in 1930. Likewise the second brood in 1929 was quicker than that in 1930, the first in 1930 was quicker than the second in 1930 and the first in 1930 was slower than the second in 1929.

In this connection the temperature during these periods (May-June and July) is interesting and will be studied in more detail at a later date. The broods, mean quickest life cycle (i.e. the time taken from when a newly emerged and mated midge commences to oviposit until the appearance of her first progeny), and the excess or deficit of temperature compared with the average are set out in Table II.

Table II. *Duration of life cycle of Rhabdophaga heterobia compared with temperature.*

Brood	No. of expts.	Dates of oviposition and first emergence	Mean of quickest life cycle in days	Standard error	Excess or deficit in temp. ° F.	Average temp. (51-52 years)
First 1929	4	May 10-13 to June 20-25	40.75	±1.601	-1.4	54.5
First 1930	15	May 10-18 to June 19-28	39.53	±0.654	+1.7	54.5
Second 1929	4	June 24-26 to July 29-August 3	35.25	±1.315	+0.7	60.6
Second 1930	11	June 22-24 to August 2-10	44.27	±0.810	-1.6	60.6

All the midges of the same family do not emerge on the same day, and this duration of flight was found to vary from 4 to 29 days in different families. This variation does not necessarily depend on the number of individuals in the family, as can be seen from the examples shown in Table III.

Table III. *Duration of flight period and size of family.*

Family	No. of individuals	Duration of flight in days	Family	No. of individuals	Duration of flight in days
1	102	6	6	71	20
2	98	13	7	59	11
3	75	14	8	57	8
4	75	7	9	52	12
5	71	8	10	46	29

All the experiments were carried out under the same conditions as far as possible. The consequence of this variation in length of life cycles (since all the eggs are laid within 12 hours) between individuals of the same family is the overlapping of the generations.

An important feature in the bionomics of *R. heterobia* is that, although reproduction is sexual, unisexual families are the rule. Yet both sexes are normally to be found in a single gall owing to oviposition by more than one female. Thus random samples of galls produce populations which show normal sex ratios of 42-48: 58-52 (7).

The occurrence of hermaphrodite forms is interesting. Two such forms¹ have been seen in this species, one in 1928 and the other in 1933. Both individuals possessed normal female antennae and normal male genitalia. Altogether just over 19,000 *R. heterobia* have been examined. It is worth noting that when studying *Dasyneura alopecuri* (9) a similar occurrence was found once in 55,000 individuals, but in this case while the individual had normal male antennae it had an ovipositor. One other similar case in gall midges has been observed, i.e. in *Mayetiola phalaris* (10), and this individual had male antennae and an ovipositor. Unfortunately it has not been possible to recognise any of these specimens when alive.

The variation in numbers of antennal segments in *Rhabdophaga heterobia* has been studied (11) and found to depend on the food supply of the larvae.

IV. THE FAUNA OF THE GALLS.

The galls are ideal places for the hibernation of various insects and other animals, and the following is a list of what is considered to be typical of the hibernating fauna: Coleoptera—*Chalcoides* (*Crepidodera*) *chloris* Foudr., *Plectroscelis concinna* Marsh, *Coccinella 2-punctata* Linn.: Mollusca—*Helix nemoralis* or *hortensis* (young): woodlice—*Philoscia muscorum* (Scopoli): spiders—*Trachygynatha dentata* Wid., *Epeira cornuta* Clerk, *Oedothorax fuscus* Bl., and *Enidia bituberculata* Wid. All the above were found in galls collected in Somerset on November 23rd, 1927. I am indebted to Dr A. Randell Jackson and Mr H. C. F. Newton who identified the spiders and beetles respectively.

In addition, one frequently finds *Anthocoris nemorum*, which sucks the larvae in the galls and later the midges as they emerge. There are also aphid eggs, the predaceous larvae of a midge *Lestodiplosis* sp., which may be *L. heterobiae* Barnes, and the larvae of external parasites of *Rhabdophaga heterobia* such as those of *Pseudotorymus medicaginis* Mayr.

The following birds have been observed pecking at the button galls and presumably feeding on the insects contained therein—song thrush (*Turdus musicus* Linn.), blackbird (*Turdus merula*, Linn.), and various kinds of tits (*Parus* spp.). The latter probably scatter as much of the contents of the gall as they eat and so help to ensure the continuance of the midge, as the larvae can live and develop in the soil as well as in the gall itself.

V. SIZE OF POPULATION OF *RHABDOPHAGA HETEROBIA* AND ITS PARASITES, 1928–33.

The midge and its parasites have been reared each year from samples of 500 galls collected the previous October–November. Owing to the method of sampling no direct evidence of fluctuations in extent to which the crop has been attacked has been obtained. But the actual numbers of insects (midge and parasites) reared shows how the infestation might have varied. The average

¹ A third hermaphrodite was reared in 1934, in this case however the individual possessed normal male antennae and a normal ovipositor.

figures are expressed diagrammatically in Fig. 1. Table IV gives the numbers of midges and hymenopterous parasites reared from the standard sized samples over the period 1928-33. The figure for the year in each case denotes the year the insects emerged: the sample was collected the previous year.

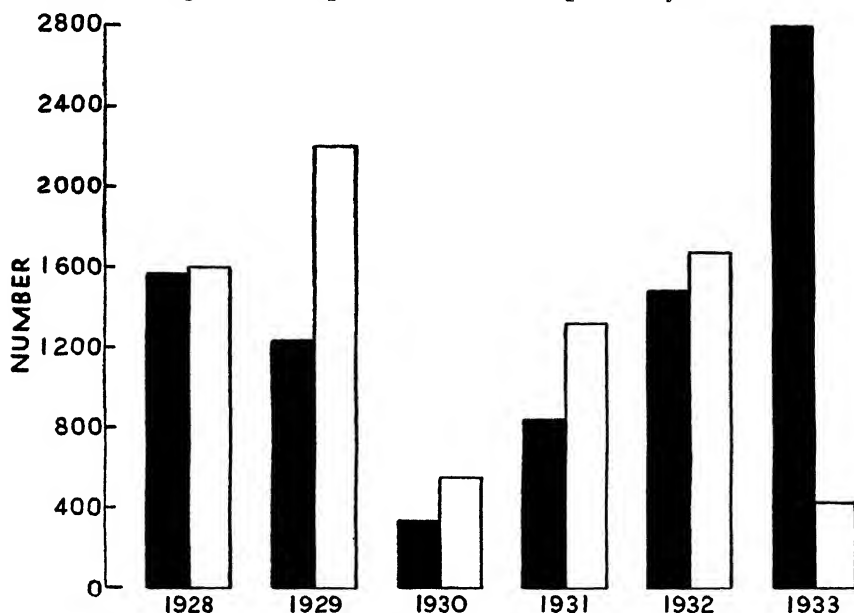


Fig. 1. Average numbers of *Rhabdophaga heterobia* (solid) and parasites (open) emerging from 500 galls, 1928-33.

Table IV. *Population of 500 galls of Rhabdophaga heterobia*, 1928-33.

Year	Total midges	Sex ratio	Total hymenopterous parasites	Parasitism %	Total insects
1928*	1573	42 : 58	1607	51	3180
1929	1235	43 : 57	2204	64	3439
1930 (i)	450	44 : 56	609	58	1059
(ii)	329	48 : 52	377	53	706
(iii)	244	45 : 55	682	74	926
1931 (i)	906	43 : 57	1470	61	2376
(ii)	867	42 : 58	1198	58	2065
(iii)	747	39 : 61	1302	64	2047
1932 (i)	1390	39 : 61	1632	54	3022
(ii)	1370	38 : 62	1589	54	2959
(iii)	1679	35 : 65	1766	51	3445
1933 (i)	2798	42 : 58	443	14	3241
(ii)	2633	47 : 53	350	12	2983
(iii)	3000	42 : 58	492	14	3492

* 488 galls comprised this sample, in all other cases 500 galls constituted a sample.

It will be seen at once that the total number of insects reared from the 500 gall samples was approximately 3000 in the years 1928, 1929, 1932 and

1933, but that in 1930 only about 1000 or one-third the usual number were reared and in 1931 only about 2000 or two-thirds the usual number appeared. The suggested reason for this reduction in total population is put forward below. However, it can be presumed that 3000 insects are the normal population obtainable from 500 galls. It is likely that this number is dependent on the food supply. Whereas in 1928, 1929 and 1932 the population was made up of roughly equal numbers of host midge and its parasites, in 1933 there was a large preponderance of midges and a corresponding decrease in numbers of parasites. Nearly twice as many midges as normal emerged, while just over one-quarter the usual number of parasites appeared. In spite of this striking change in make-up of the population, the total number was remarkably similar to that of 1928, 1929 and 1932. This sudden or cataclysmic reduction in numbers of parasites is further dealt with in section VI, "Relative parasitism of the midge."

The great reduction in numbers both of parasites and midges in 1930 and the consequent slow approach to normal as shown by the figures for 1931 are explicable as follows. In the summer of 1929 there was a serious drought and the willows stopped their terminal growth and started forming the winter buds, i.e. those which would remain over winter, considerably earlier than usual. Perhaps the descent of the sap had started much earlier than usual, and in any case the amount rising to the terminal shoots was seriously curtailed. The larvae of *R. heterobia* obtain their nourishment at the terminal buds and naturally the parasites depend on their host's food supply. It is reasonable to suppose that, owing to this shortage of sap, both the midge larvae and the parasites were short of food, and instead of each gall being able to support adequately roughly six insect larvae they could only support two the first year, i.e. 1929-30, and four the next year, 1930-1. It will be noticed that it took two years before the normal population (3000) was reached again; this means several broods, as only the over-wintering brood was studied.

As one collected the galls in 1929 the impression received was that they were smaller than usual though slightly less numerous. It was not until 1931 that they appeared as large as when collected in 1927 and 1928.

The effect of this drought on the plants was also very obvious. The willows in 1930 were badly stunted and only about one-third their normal height at the end of the year. The following year they were still much shorter than usual, roughly two-thirds their normal height, and in 1932 they were still slightly shorter than normal, but in this last case their abnormality was probably due in some part to the non-cultivation of the willow bed. In 1932 the owners ceased to look after the field, merely cutting the crop but not attempting to weed at all.

Another interesting observation is that the total numbers of midges per sample were increasing, the numbers of males appearing to be decreasing; e.g. in 1930, the year with the lowest midge population, the sex ratio varied from

44:56 to 48:52. As the numbers increased in 1931 the sex ratio varied from 39:61 to 43:57, and in 1932 when the midge population was apparently up to normal the sex ratio had decreased from 35:65 to 39:61. More data are required before this can be explained, as in 1933 the sex ratio was 42:58 to 47:53. However, it must be remembered that this species of midge produces unisexual families. It may be that in cases of overcrowding or any other deleterious factors the males are the first to suffer lethally. There is some evidence to support this, as previous work (8) on the effect of temperature on emergence showed that while females of another species of midge, *Dasyneura alopecuri*, were more affected by non-lethal factors, males were more affected by lethal ones.

VI. RELATIVE PARASITISM OF THE MIDGE.

(i) Identification.

I am indebted to Dr Ferrière of the Imperial Institute of Entomology for identifying some of the parasites for me. He reports finding the following in the tube of parasites which emerged in 1932: Chalcidoidea, Tetrastichini, *Aprostocetus ciliatus* Nees ♀ ♂ and *Tetrastichus inunctus* Nees ♀; Proctotrypoidea, Platygasterinae, *Synopeas gallicola* Kieff. ♀ ♂; and Torymidae, *Pseudotorymus medicaginis* Mayr ♀.

In addition to these, two further parasites have been reared in 1933, namely a *Platygaster* species and *Inostemma walkeri* Kieff. The latter, Dr Ferrière tells me, is that British species which Walker named *I. boscii*, but which is different from the real *boscai* Jur.

(ii) Methods and results.

The methods used were the same as those described in the first two papers in this series (1, 2).

The species of parasites reared have only been identified, and up to the present no attempt has been made to find out how prevalent each species has been from year to year. All the material has been kept for this purpose. In addition, before a true idea of the situation can be obtained, the interrelationships of the various parasites must be discovered.

However, the gross relative parasitism can be seen in Table IV, and the average yearly relative parasitism of the over-wintering brood is set out graphically in Fig. 2.

It can be seen that the relative parasitism has been more or less constant over the period studied, varying between 51 and 64 per cent., with the exception of 1933. This is in marked contrast to the comparatively large fluctuations obtained when studying the wheat blossom midges (1) and the meadow foxtail midge (2). But in these each successive brood of midges was being studied, whereas in this case the broods intervening between successive over-wintering ones have not been under observation. It appears possible that the relative

parasitism in these latter broods would reveal similar larger fluctuations, unless of course a stabilised condition between host midge and total number of parasites has been reached in this particular field of willows.

Since the fall in total numbers of insects in the samples in 1930 affected the numbers of midges and parasites to the same extent, the relative parasitism remained about the same. On the other hand, in 1933 there was a large de-

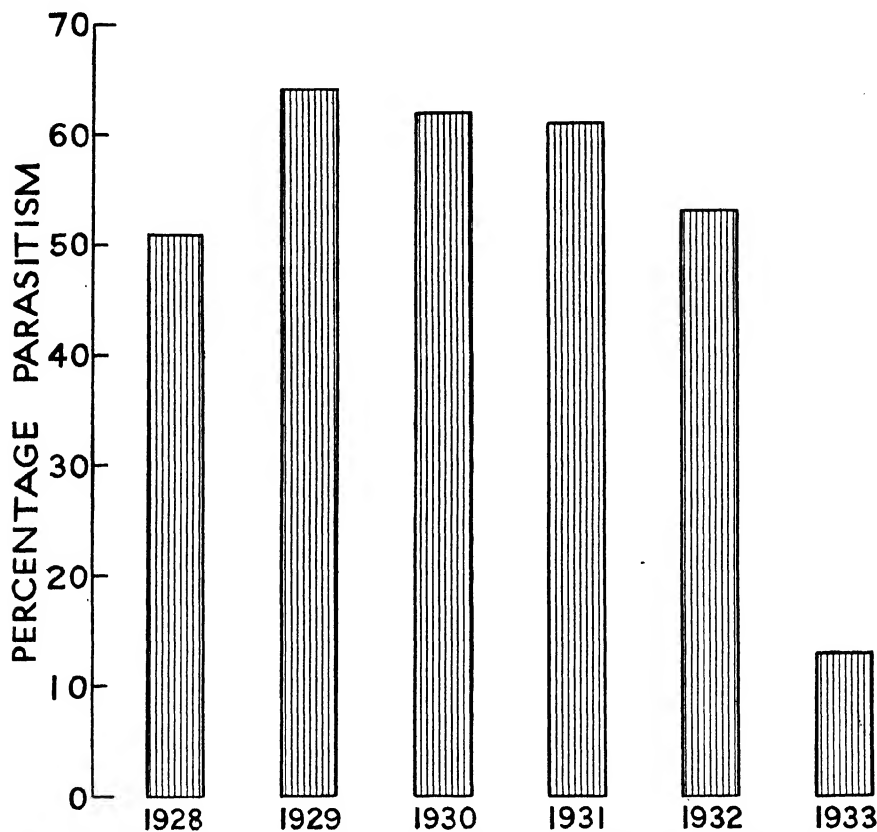


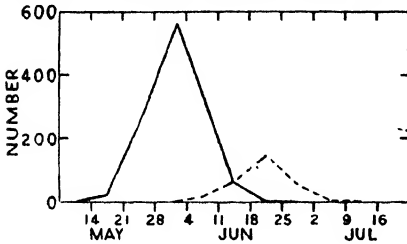
Fig. 2. Average relative parasitism of over-wintering brood of *Rhabdophaga heterobia*, 1928-33.

crease in the extent of the relative parasitism as the numbers of parasites emerging decreased and the numbers of midges increased. A reversal in the relative times of emergence of host and parasites, as has already (2) been suggested when dealing with another species of midge, *Dasyneura alopecuri*, is a possible explanation of this sudden fall in numbers of parasites. In Fig. 3 one set of normal emergence curves of *D. alopecuri* and its parasites are shown (A). In addition the abnormal emergence curves which represent the emergences occurring in 1928 are shown (B), together with the emergence

curves of the next brood which took place the subsequent year 1929 (C). It will be seen that normally the crest of emergence of the host midge is before

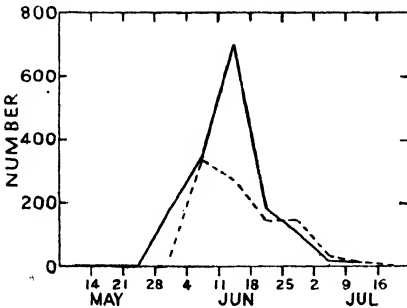
A. NORMAL EMERGENCE 1930

TOTAL MIDGES 1244
 " PARASITES 284
 RELATIVE PARASITISM 19 PER CENT



B. ABNORMAL EMERGENCE 1928

TOTAL MIDGES 1588
 " PARASITES 979
 RELATIVE PARASITISM 38 PER CENT



C. RESULT OF ABNORMAL EMERGENCE 1929

TOTAL MIDGES 4748
 " PARASITES 114
 RELATIVE PARASITISM 2.3 PER CENT

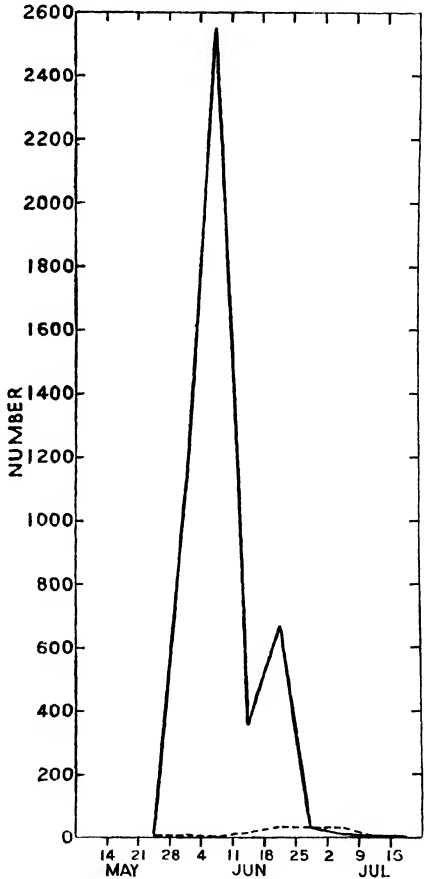


Fig. 3. Effect of alteration in relative times of emergence of *Dasyneura alopecuri* (full line) and its parasites (dotted line). A, normal emergence of *D. alopecuri* and its parasites; B, abnormal emergence, parasites relatively early; C, result of abnormal emergence, viz. increase of midges and fall in number of parasites in the following brood.

that of the parasites. In 1928, however, the peak of the parasites was ahead of that of the midges. As a probable direct consequence of this the numbers of midges increased from 1588 in 1928 to 4748 in 1929. Similarly the numbers of parasites fell from 979 in 1928 to 114 in 1929. The sudden fall in numbers

of parasites of *Rhabdophaga heterobia* in 1933 and the great increase in numbers of *R. heterobia* are possibly due to some such reversal in emergence dates of the previous generation. But since the over-wintering brood of this midge was the only one studied, we have no corroboration of this hypothesis.

VII. EMERGENCE.

Table V shows the dates of the actual first emergence, the peak of emergence, the number of days to reach the peak and the date of the last emergence of *R. heterobia* in the samples as kept in the outdoor insectary during the period 1928-33 inclusive.

Table V. *Dates of actual first emergence, peak of emergence, number of days to reach peak and the dates of the last emergence of Rhabdophaga heterobia, 1928-33.*

Year	Date of first emergence	Date of peak of emergence	Days to reach the peak	Date of last emergence
1928	April 29	May 28	30	July 9
1929	May 10	June 1	23	August 8
1930 (i)	" 10	" 2	24	July 24
(ii)	" 12	" 2	22	" 5
(iii)	" 14	" 2	20	" 2
1931 (i)	" 2	May 27	26	" 16
(ii)	" 6	" 27	22	" 16
(iii)	" 11	" 28	18	" 16
1932 (i)	" 17	June 13*	28	August 7
(ii)	" 18	" 13*	27	c. " 28
(iii)	" 16	" 13*	29	" 15
1933 (i)	April 26	May 29	34	c. July 17
(ii)	" 27	" 22	26	" 3
(iii)	" 25	" 22	28	" 11

* In each sample in 1932 there was a definite crest of emergence on June 2nd followed by a larger but abnormal crest on June 13th, a day after the weekly spraying for the year was started. It had been inadvertently omitted up to then owing to pressure of other work.

The range of first emergence is from April 25th to May 18th (23 days), while that of the peaks is from May 22nd to June 13th (22 days). Within samples of the same year the corresponding range between dates of first emergences has been 9 days in 1931, 4 in 1930, 2 in 1932 and 2 in 1933. In the case of the peaks it has been 1 day in 1931 and 7 days in 1933; in 1930 and 1932 the peaks occurred in the three samples on the same day. The time which lapses between the first emergence and the peak varies from 18 to 34 days if we consider the whole period of six years. The variation within any one year is much less, about one-half, e.g. 20-24 days in 1930, 18-26 days in 1931, 27-29 days in 1932 and 26-34 days in 1933.

Comparing these figures with those obtained when studying *Dasyneura alopecuri* (2), it will be seen that the dates obtained for the different samples of any one year agree more closely in the case of *Rhabdophaga heterobia* than in that of *Dasyneura alopecuri*. This difference between the two species may be due in part to the different over-wintering positions of the larvae. In the grass species, *D. alopecuri*, the larvae remain in the florets when they

Table VI. *Weekly emergence of Rhabdophaga heterobia and its parasites, 1928-33. The upper rows of figures refer to the midges, while the lower refer to the parasites. (Note. The true crest of emergence in 1932 was probably in the week May 28th-June 3rd.)*

Year	Date and size of sample	April 23-29	April 30-May 6	May 7-13	May 14-20	May 21-27	May 28-June 3	June 4-10	June 11-17	June 18-24	June 25-July 1	July 2-8	July 9-15	July 16-22	July-August later	Total host and parasites	Parasitism %
1928	1. xi. 27, 488 galls	1	33	102	84	330	590	236	156	31	8	1	1	—	—	1573	51
1929	31. x. 28, 500 galls	—	—	—	5	132	520	228	547	354	143	92	24	—	—	1607	64
1930	30. x. 29, 500 galls, A	—	—	—	7	75	234	402	689	431	100	73	68	52	13	2204	58
	B	—	—	—	2	2	8	60	7	3	1	2	2	1	—	609	53
	C	—	—	—	1	40	128	116	10	2	0	1	—	—	—	329	74
1931	31. x. 30, 500 galls, A	—	—	19	23	322	282	132	379	152	25	3	2	1	—	682	61
	B	—	—	9	18	310	307	117	507	159	40	54	79	41	19	1470	58
	C	—	—	5	13	215	286	139	397	101	48	50	87	62	17	1198	64
1932	30. x. 31, 500 galls, A	—	—	—	2	5	87	300	463	116	42	75	124	56	21	1302	54
	B	—	—	—	24	83	303	293	505	117	35	10	3	0	17	1390	54
	C	—	—	—	4	2	26	137	457	491	371	70	52	5	17	1632	54
1933	1. xi. 32, 500 galls, A	6	80	196	485	935	902	151	35	2	2	1	3	5	0	2798	14
	B	4	34	138	439	1034	715	172	49	36	23	13	3	5	0	443	12
	C	5	38	146	467	1120	689	101	52	33	16	14	7	0	0	350	14
					2	30	95	478	33	3	6	3	1	0	0	3000	
					1	42	104	137	57	62	36	36	12	4	1	492	

fall to the ground and so will find themselves in all kinds of exposed and unexposed positions, under leaves, etc. But in the other species, *Rhabdophaga heterobia*, the larvae remain in the buttons or galls on the tips of the willow shoots and so are all exposed to the same conditions, i.e. atmospheric conditions as opposed to varying soil surface conditions as is the case in *Dasyneura alopecuri*.

It is hoped to study the factors controlling day to day emergences in detail at some later date.

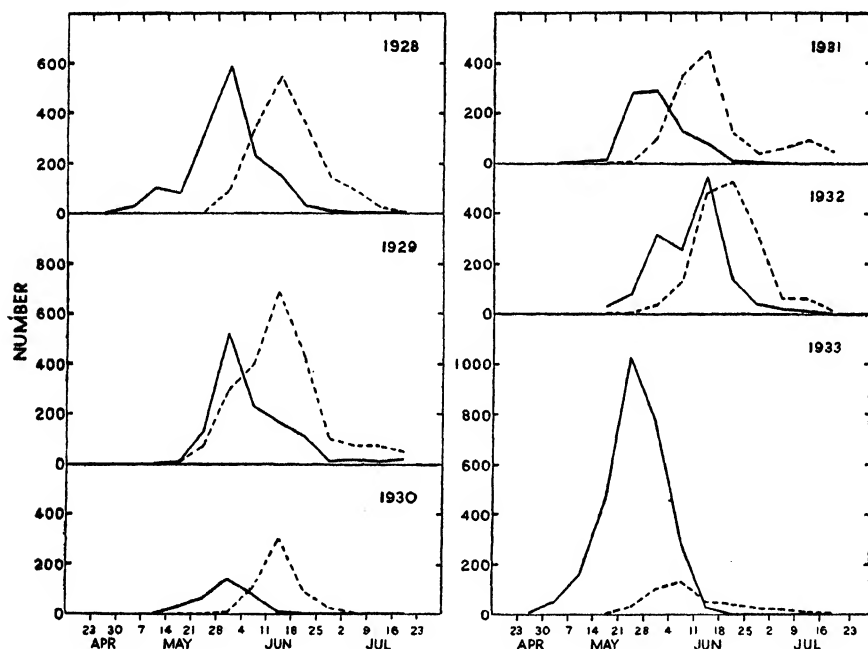


Fig. 4. Weekly emergence of *Rhabdophaga heterobia* (full line) and its parasites (dotted line), 1928-33.

The dates and figures in Table VI show the weekly emergences of the midge and its parasites. The top row of figures in each case refers to the midge, while the lower row refers to the parasites. The peaks are in heavy type. It will be seen that a weekly crest of emergence has occurred five times in the week May 21st to 27th, five times in the week May 28th to June 3rd, once in the week June 4th to 10th, and three times in the week June 11th to 17th. But in contrast to what happened in the case of *D. alopecuri* (2) there is little difference between the samples of any one year.

The emergence of the parasites has varied less, the crest occurring in the week June 4th to 10th three times (1 year), in the week June 11th to 17th eight times (4 years), and in the following week, June 18th to 24th three times

(1 year). No variation occurred in the samples of any one year. Usually the crest of emergence of the parasites occurs in the second or third week after that of the host midge.

Fig. 4 shows the average weekly emergence numbers of the midge and its parasites over the period 1928-33. The figures have been obtained by averaging the three samples of each year. The constancy of the date of the weekly crest of emergence of the midges and the parasites is very noticeable.

The emergence of both the midge and the parasites drags out a long while. This may be so in the field, it may not. Under insectary conditions of keeping water evaporating through the sample and spraying the top of it once a week, one obtains definite minor peaks occurring the day after such spraying. Table VII shows this.

Table VII. *Showing effect of spraying top of sample weekly during the emergence period. Evening of spraying marked with asterisk.*

Date (June)	No. of midges emerging		No. of parasites emerging	Date (June)	No. of midges emerging		No. of parasites emerging
	♂	♀			♂	♀	
3*	53	45	61	14	8	1	30
4	20	35	55	15	7	9	107
5	2	4	13	16*	3	2	58
6	0	3	25	17	34	44	221
7	17	32	81	18	15	14	115
8	11	14	26	19	10	10	93
9*	7	21	98	20	8	6	71
10	35	37	104	21	2	0	36
11	11	7	95	22	0	1	13
12	4	3	39	23*	0	4	47
13	7	15	139	24	26	17	56

This indicates how beneficial a shower of rain might be in the field.

It has been shown previously (8) that considerable emergences may take place at intervals during May if the weather is favourable, but do not under adverse weather conditions. It was then stated that extra cold given to the larvae during the winter tended to retard the initial emergence, and so causes the crest of emergence to be reached sooner. Also that cold affected the larvae of *Dasynura alopecuri* less than those of *Rhabdophaga heterobia*.

These facts, together with the appearance of minor crests after the main crest, lend colour to a view that is being built up and strengthened by these studies of gall midges. This view is that the larvae require a certain more or less fixed amount of temperature to develop from fully fed larvae into adults; when such amount of temperature has been made available to the insects, then under given favourable conditions from day to day emergences will take place. On the other hand, however, favourable day to day conditions may occur before such amount of requisite temperature has been received, emergences will not occur. The actual day to day emergences seem to depend upon day to day conditions to some extent, but finally, when the insect is ready, the urge to emerge is overwhelming and as a result the crest of emergence is

nearly constant. Attempts are being made to obtain a rough index figure for this necessary amount of temperature without studying the threshold developmental temperatures for each stage. If such an index can be obtained one of the objects of this investigation will have been achieved.

VIII. SUMMARY.

1. This is the third of a series of studies on the fluctuations of insect populations in the field and is concerned with the gall midge *Rhabdophaga heterobia* H.Lw.

2. The time taken by this midge to complete a life cycle is discussed, and notes are given of occurrences of hermaphrodite individuals in gall midges.

3. A list is given of various spiders, beetles and other animals which have been found hibernating in the galls of this midge.

4. The changes in populations of the midge and its parasites in the overwintering generation have been traced over a period of six years, 1928-33. A drought which seriously damaged the growth of the willows is claimed to have caused a great diminution in numbers of both host midge and its parasites. It is pointed out that the numbers did not re-attain the normal until the third year after the drought.

5. The relative parasitism of the midge for this period of six years is given. A sudden fall in the relative parasitism in 1933 is discussed and compared with a similar occurrence noted in the second of this series of studies.

6. The emergence of the midge and its parasites is set forth. It is shown that the date of the peak of emergence of the midge is nearly constant whether the initial emergences are earlier or later than normal.

IX. ACKNOWLEDGMENTS.

I am indebted to the persons mentioned in the context for their help in diverse directions and also to Dr C. B. Williams and Dr A. D. Imms for discussing various points with me. I wish to acknowledge my thanks to Mr A. D. Dunkley who has again prepared the figures.

REFERENCES.

- (1) Barnes, H. F. (1932). "Studies of fluctuations in insect populations. I. The infestation of Broadbalk wheat by the wheat blossom midges (Cecidomyiidae)." J. Anim. Ecol. 1, 12-31.
- (2) Barnes, H. F. (1933). "Studies of fluctuations in insect populations. II. The infestation of meadow foxtail grass (*Alopecurus pratensis*) by the gall midge *Dasyneura alopecuri* (Reuter) (Cecidomyiidae)." J. Anim. Ecol. 2, 98-108.
- (3) Barnes, H. F. (1930). "On the resistance of basket willows to button gall formation." Ann. Appl. Biol. 17, 638-40.
- (4) Barnes, H. F. (1931). "Further results of an investigation into the resistance of basket willows to button gall formation." Ann. Appl. Biol. 18, 75-82.
- (5) Magerstein, C. (1932). "Bejdomorka ržicetvorná (*Rhabdophaga rosaria* H. Loew.) na Konopíně (*Salix viminalis*)." Ochr. Rost. 12, 86-9.
- (6) Barnes, H. F. (1929). "Button top of basket willows." J. Min. Agric. 65-71.

- (7) **Barnes, H. F. (1931).** "The sex ratio at the time of emergence and the occurrence of unisexual families in the gall midges (Cecidomyiidae: Diptera)." J. Genet. 24, 225-34.
- (8) **Barnes, H. F. (1930).** "On some factors governing the emergence of gall midges (Cecidomyiidae: Diptera)." Proc. Zool. Soc. Lond. 381-93.
- (9) **Barnes, H. F. (1930).** "On the biology of the gall-midges (Cecidomyiidae) attacking meadow foxtail grass (*Alopecurus pratensis*), etc." Ann. Appl. Biol. 17, 339-66.
- (10) **Barnes, H. F. (1927).** "A new gall midge (*Mayetiola phalaris* sp.n.) reared from *Phalaris arundinacea*." Z. Angew. Ent. 13, 375-7.
- (11) **Barnes, H. F. (1932).** "A study of the segmentation of the antennae in gall midges (Cecidomyiidae)." Proc. Zool. Soc. Lond. 323-34.
- (12) **Roebeck, A. (1934).** "A general account of the pests on willows at Syston, Leicestershire." J. Anim. Ecol. 3, 182-6.

ON THE BIOLOGY OF SOME SPECIES OF *LONGITARSUS* (COL., CHRYSOM.) LIVING ON RAGWORT.

By H. C. F. NEWTON, B.Sc., A.R.C.S.,

Entomology Department, Rothamsted Experimental Station, Harpenden, Herts.

1. Introduction.

It appears from the literature that the species of *Longitarsus* recorded from ragwort (*Senecio jacobaea*) or its ally the groundsel (*S. vulgaris*), are *L. dorsalis*, F., *L. suturellus*, Duft., *L. senecionis*, Bris., *L. jacobaeae*, Wat., *L. ochroleucus*, Marsh., and *L. gracilis*, Kutsch.; the last named species is also recorded by Bedel from *Tussilago*. In addition *L. succineus*, Foudr., has been associated with various composites, such as *Eupatorium cannabinum* and *Chrysanthemum* spp. but, apparently, never before with *Senecio*.

During the seasons 1928–29 the author was engaged in work on *Tyria jacobaeae* for ragwort control in New Zealand and this entailed frequent visits to the "Brecklands" of south-western Norfolk for the collection of its pupae, etc., for transit to New Zealand. It was on these occasions and less frequently since, when opportunity offered, that the following observations on some *Longitarsus* beetles living on ragwort were made. Not only because of the interest attached to knowledge of the larval stages of such a variable genus as *Longitarsus*, but because also of its bearing on the insect-plant relations of *Senecio jacobaea*, this note has been written.

In all, some seven species of *Longitarsus* were met with, viz., *L. jacobaeae*, *L. dorsalis*, *L. succineus*, *L. gracilis*, *L. exoletus*, L., *L. luridus*, Scop., *L. melanocephalus*, All., but of these the last three were not associated with *Senecio*. Of the rest, and it is with them that this note deals, *L. jacobaeae*, *L. gracilis* and *L. succineus* are stated to be generally abundant throughout the kingdom, while *L. dorsalis* is not common and apparently confined to the southern half of England.

I have pleasure in acknowledging help I have received from Mr. J. R. le B. Tomlin and Mr. H. Britten of the Manchester Museum in confirming the identifications.

2. *Longitarsus jacobaeae*, Wat.

This is one of the most abundant of the *Longitarsus* species and is stated to occur generally throughout the kingdom. It is well distributed over the ragwort areas of the "Brecklands" of south-western Norfolk. Bedel gives its distribution as "river banks, littoral cliffs abundant on the Channel coasts, less frequent in the interior and rather generally confined to the lengths of the great river-beds, e.g., Seine and Marne." The "Brecklands" are, however, essentially dry regions.

Fowler recognises a darker form var. *rufescens* varying from ferruginous to a clear red in exceptional cases; this variety was occasionally present.

Food-plants.

Ragwort (*Senecio jacobaea*) appears to be the only recorded food-plant. In the laboratory the beetles were fed from the beginning of August to the end of November on the following plants. At the end of this period all survived on *S. jacobaea*, 80 per cent. on *S. aquaticus*, 50 per cent. on *S. sylvaticus*, 30 per cent. on *S. vulgaris*, 50 per cent. on cultivated sunflower (*Helianthemum* sp.), none on the aster (*Callistephus* sp.), Michaelmas daisy (*Aster* sp.), marguerite (*C. leucanthemum*) and golden rod (*Solidago virgaurea*). Of the last three plants, the foliage of Michaelmas daisy and marguerite were only slightly nibbled, the other was untouched.

Life-history.

The new generation of beetles was first seen at the end of July, the numbers reaching a maximum in late August. After emergence copulation takes place at once and egg-laying follows soon afterwards. The first eggs were seen in mid-August. They are laid in the soil. Those laid in late summer hatch in about a month or under, but later ones do not hatch till the following spring, so that the winter is passed in both egg and larval stages. Development goes on at the expense of the root system of the host, but although the larvae may be numerous, the growth of the plant is not noticeably affected. Fully grown larvae occur from June onwards, pupation follows in the usual earthen cell, lasts for a fortnight to three weeks, and the newly emerged adults, as stated above, begin to be seen during the latter half of July. There appears to be but one generation a year.

It is notable that *L. jacobaeae*, by passing the winter in the developmental stages, differs from the majority of flea-beetles studied by the writer. In this respect, however, it agrees with *Psylliodes chrysocephala* (cabbage-stem flea-beetle). This fact has a bearing on its effectiveness as a controlling agent of the growth of its host-plant. It means that the appearance of the beetles does not coincide with that of the young ragwort plants. They are well established by the time of the mass emergence of the beetles and so escape the early attack that is so devastating in the case of the Cruciferae-eating flea-beetles.

Description of the Developmental Stages.

The egg of *L. jacobaeae* is elongate, oval, with rounded ends, and measures 0.66 mm. long and is rather less than half as broad. It is yellow in colour, becoming darker before hatching. Its surface sculpturing is similar to that of other flea-beetle eggs and consists of a network of polygonal pits (fig. 1).

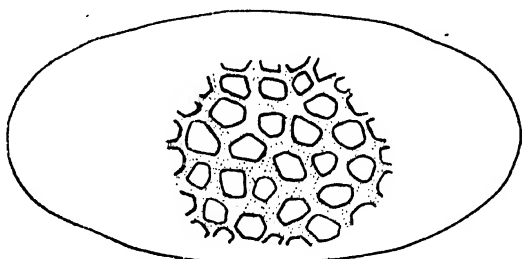


Fig. 1. *L. jacobaeae*, Wat.: egg in outline ($\times 95$) with part of surface highly magnified ($\times 475$) to show sculpturing.

The young larva, which bites its way out of the egg-shell—a leisurely proceeding taking some hours, is about 1.5 mm. long and 0.25 mm. in width. The head, prothoracic shield and anal plate are dark greyish brown, the head being darkest. The legs and segmental plates faintly brown. The arrangement of the segmental plates and setae is essentially the same as in the fully grown larva, but relative to the size of the young larva the segmental plates are much larger than in later life, so that they almost completely cover the segments. The head is also proportionately large.

The fully grown larva is some 6 mm. long and just over 1 mm. across. It is white in colour, elongate in form, similar to that of *L. dorsalis* (fig. 7) but somewhat less slender and more robust. The head-capsule is dark brown, anal plate and prothoracic shield brown, and legs light brown. The three short stumpy pairs of thoracic legs shows the usual five segments. The first eight abdominal segments are similar, the

ninth carries the anal plate above, and beneath the anal proleg containing the anal opening on the reduced tenth segment. One thoracic and eight abdominal spiracles are present.

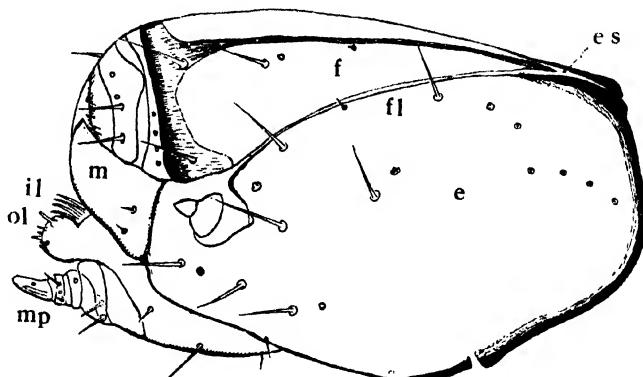


Fig. 2. *L. jacobaeae*, Wat.: side view of head-capsule to show arrangement of setae, $\times 190$; *mp*, maxillary palp; *ol*, outer lobe of maxilla; *il*, inner lobe; *m*, mandible; *f*, frons; *fl*, fronto-lateral suture; *e*, epicranium; *es*, epicranial suture.

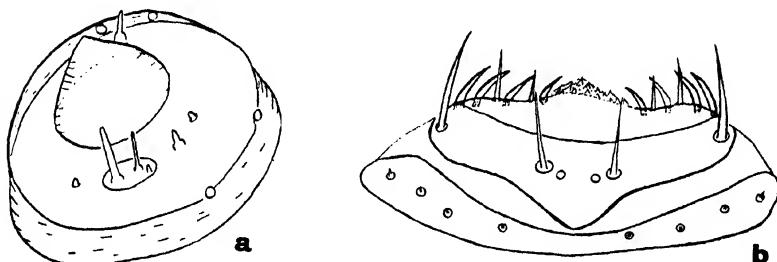


Fig. 3. *L. jacobaeae*, Wat.: *a*, right antenna from above—schematic, $\times 900$; *b*, labrum and clypeus, $\times 475$.



Fig. 4. *L. jacobaeae*, Wat.: side view of prothorax, $\times 120$; *ps*, prothoracic shield.

The head-capsule (fig. 2) is well chitinated above, weakly beneath, and shows the fronto-lateral and epicranial sutures and other usual features of flea-beetles. The arrangement of the setae is seen from the figure. The sensory organs of the antennae are shown in schematic form in fig. 3, *a*; the labrum in fig. 3, *b*. The

strong, five-toothed mandibles bear two strong spines (occasional specimens have three) on their inner surface above the molar area and externally bear two setae; the maxilla with its well armed galea and lacinia and segmented palps presents no unusual features. Dorsally the prothoracic shield (fig. 4) covers the prothorax; it carries an anterior row of 8 and a posterior row of 6 setae, and has a median suture. Fig. 5A shows the arrangement of the segmental plates on the meso-thorax which,

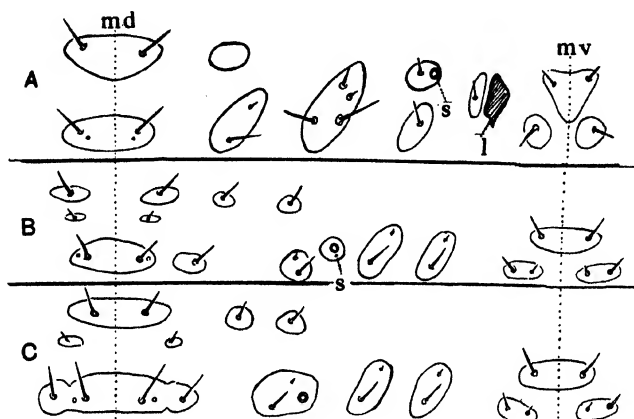


Fig. 5. *L. jacobaeae*, Wat.: map of arrangement of chitin plates and setae on A, meso-thorax; B, abdominal segments I-VII; C, abdominal segment VIII; md, mid-dorsal line; mv, mid-ventral line; l, leg; s, spiracle.

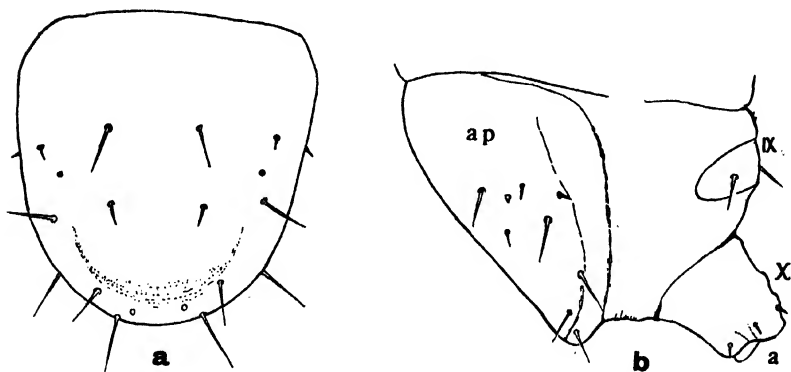


Fig. 6. *L. jacobaeae*, Wat.: a, dorsal view of anal plate; b, side view of posterior end of larva, $\times 80$; ap, anal plate; a, anus; IX and X, ninth and tenth abdominal segments.

except for the presence of the spiracle, is the same as that on the metathorax; fig. 5B that on abdominal segments 1-7; fig. 5C that on the 8th abdominal segment. The anal plate (fig. 6, a) is broad, rounded and slightly depressed apically.

A large number of larvae of *L. jacobaeae* have been examined under high power, and it appears that the structural characters outlined in this note are essentially

constant. The same applies to *L. dorsalis*, but in this case fewer larvae have been compared. One point may, however, be mentioned. In sorting them over, the larvae of *L. jacobaea* fell into two groups of approximately equal numbers depending on a slight difference in the shape of the anal plate. This in the one case was of a narrower more elongated form, in the other a slightly broader type. It is possible that this may be a sexual difference.

The Pupa and Prepupa.

The prepupa assumes the normal recurved shape and builds up the earthen cell in which pupation takes place.

The pupa is white and of the usual flea-beetle form. The chaetotaxy is as follows: Head, 6 setae, 3 in a vertical line between the eyes on each side of middle line; often the median seta is duplicated. Thorax, 10 setae; 2 pairs median, 1 pair antero-lateral, 1 pair postero-lateral, 1 pair medio-lateral near lateral margin; the latter setae may be duplicated. Meso- and meta-thorax, 4 setae each in a transverse line. Abdomen 8 setae in a transverse line, the external setae on each side (pleural) below the stigma. The 7th abdominal segment shield-shaped; 8th and 9th reduced, the latter bearing the brown anal horns. Legs with 2 setae, the femur at the femoro-tibial bend.

3. *Longitarsus dorsalis*, F.

This is a distinctive species which appears to have no variations, and consequently there is no confusion about the nomenclature.

Food-plants.

Senecio jacobaea and *S. vulgaris* are given by Fowler, *S. erucifolius* by Foudras, *S. erucifolius*, *S. vulgaris* and perhaps also *Erigeron canadense* by Bedel.

It was found by the writer in a field of ragwort surrounded by woodland on the edge of the "Breckland" district of south-west Norfolk in very large numbers; on the more open "Brecks" of the neighbourhood it still occurred but was much more thinly distributed. *L. jacobaeae* was also present later in the year in the same field, but in distinction from *L. dorsalis* this species occurred in great numbers over most of the "Breckland" area.

L. dorsalis was not taken in the field on plants other than ragwort, but in the laboratory it was fed on related plants for some four months. At the end of this period all the beetles had survived on *Senecio vulgaris*, *S. sylvaticus*, and *S. aquaticus*, 90 per cent. on sunflower (*Helianthemum* sp.), 75 per cent. on marguerite (*C. leucanthemum*), 50 per cent. on a cultivated Michaelmas daisy (*Aster* sp.), and 25 per cent. on golden rod (*Solidago virgaurea*).

Life-history.

Adults of *L. dorsalis* were first observed in April and could be found on the plants till July, by the end of which month they had practically all died off. Egg-laying had already begun towards the end of April and continued throughout May and June, the eggs, as with *L. jacobaeae*, being deposited in the soil. Half-grown larvae were found in August, full-grown larvae in late August and September, pupae in late September and early October, the mass appearance of the new generation occurring in that month. Unlike *L. jacobaeae*, the winter appears to be passed in the adult stage.

Description of the Immature Stages.

The egg is of similar dimensions (0.6 mm. \times 0.23 mm.) to that of *L. jacobaeae* and possesses the same type of sculpturing, but is of an orange colour.

The larva (fig. 7) differs from *L. jacobaeae* in two features which easily allow of their separation. First, the anal plate is more pointed and pear-shaped, with a depression at its tip. Second, the dorsal anterior median plates of abdominal segments 1-7 are

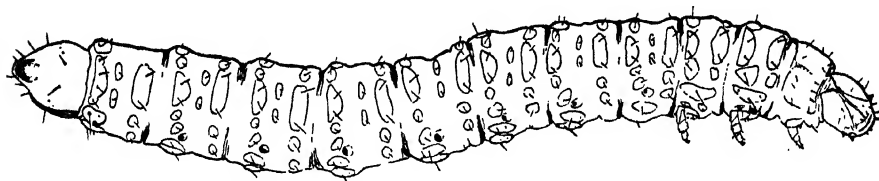


Fig. 7. *L. dorsalis*, F.: larva, half-grown, \times 30 (drawn from potash preparation).

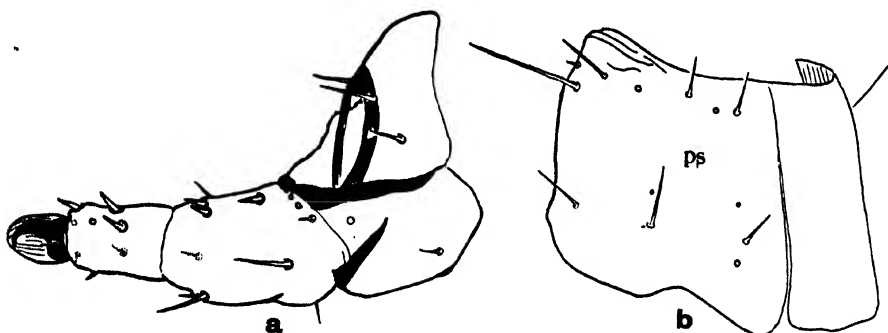


Fig. 8. *L. dorsalis*, F.: a, right prothoracic leg (ventral internal view), \times 295; b, prothoracic shield.

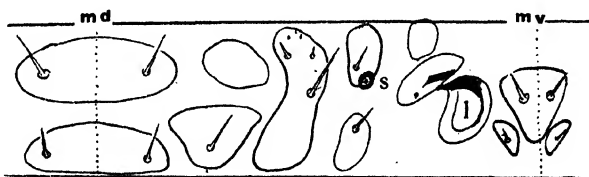


Fig. 9. *L. dorsalis*, F.: arrangement of setae on mesothorax; md, mid-dorsal line; mv, mid-ventral line; l, leg; s, spiracle.

not divided. These points together with other features of the larva are shown in figs. 8-10. A minor point of difference is in the chaetotaxy of the head-capsule. The median frontal seta is much smaller. The arrangement of the sense-organs on the antennae and the armature of the labrum is identical, however.

So far as my observations go the pupa cannot with certainty be separated from that of *L. jacobaeae*.

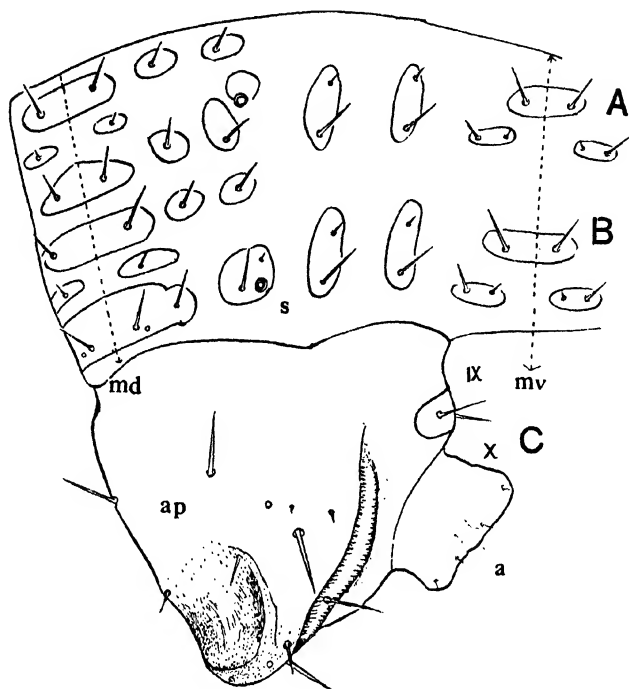


Fig. 10. *L. dorsalis*, F.: arrangement of setae on A, abdominal segments I-VII; B, abdominal segment VIII; C, posterior end of larva (segments IX and X); *md*, mid-dorsal line; *mv*, mid-ventral line; *a*, anus; *ap*, anal plate; *s*, spiracle.

4. *Longitarsus succineus*, Foudr.

This species does not appear in Fowler's Coleoptera, but in the supplementary volume (1913) *L. aeruginosus*, Foudr., is given with the statement that it is the same species as *laevis* of the British Catalogue, the *laevis* of Allard being the same as the *succineus* of Foudras.

L. aeruginosus was established by Foudras, and although Allard makes his *L. laevis* synonymic with it, Bedel treats them as distinct species. On this point Tomlin & Sharp (1912) write:—"This author's discrimination, however, does not appear to us to be very convincing, resting as it does on the difference in character of the apical cilia, which are certainly quite easily abraded; and in the comparative length of the antennae, which differ sexually. Moreover we have had British specimens discriminated as *L. aeruginosus* returned to us by Mr. Bedel himself as *L. succineus*. We therefore cannot escape the conclusion that whether the insect described as *L. aeruginosus* by Foudras is specifically valid or not, the specimens we take in this country on *Eupatorium* (the food-plant of '*L. aeruginosus*') or any other composite are all of one species—*L. succineus* Foudr." It appears therefore that the *L. aeruginosus* of Fowler's supplement does not stand; it would further seem that the description of his *L. laevis*, Duft., in Vol. IV applies to the same insect as the *laevis* of Allard, though Bedel does not regard them as synonyms.

Food-plants.

Heikertinger (1926) records the beetle from *Eupatorium cannabinum* from the end of July to the beginning of September, and from *Chrysanthemum* sp. cult. (? *indicum*) at the end of August. He regards it as an oligophage on composites, perhaps with a narrow choice, and says that although during the summer the insect is one of the commonest of the *Longitarsus* spp. in meadows and hedges, etc., there is still some uncertainty regarding its food-plants. It is found at times in large numbers remote from *Eupatorium* or *Chrysanthemum* on different plants in meadowland. Experiments with food-plants furnish indefinite results. The following composites were sometimes eaten:—*Achillea millefolia* (often refused), *Artemisia vulgaris*, *Tussilago farfara*, and *Cirsium arvense*; plants of other families often eaten were, *Symphytum officinale*, *Salvia nemorosa* and *pratensis*, *Thymus serpyllum*, *Plantago lanceolata* (preferred), *P. major*, and others. Bedel gives "Friches et coteaux secs; sur diverses Composées, *A. millefolium* (St. Clair-Deville), *Leucanthemum vulgare*, *Artemisia campestris* (Weise)." It is to be observed that Bedel separates his *aeruginosa*, Foudr., and *succinea*, Foudr.; and the former is described from *Eupatorium cannabinum* in summer and autumn, occurring in "lieux herbes très humides."

Though Heikertinger does not appear to recognise the *aeruginosus* of Foudras (regarding it as *succineus*), the difference in the habitat in the two cases seems to indicate that there are two species involved. The habitat of Heikertinger's *succineus* is given as dry.

Tomlin & Sharp are able to confirm Allard's statement that the beetle is common on *Chrysanthemum* in gardens (e.g., in Cheshire). It has been taken on *C. leucanthemum* in Devonshire, and on *Achillea millefolium* in Surrey. These authors say it is sometimes taken on *Eupatorium cannabinum* and is one of the most abundant species throughout the late summer and autumn, its range extending throughout the kingdom.

The writer found it occurring in very large numbers indeed on the ragwort areas of the Norfolk Brecklands, and its only food-plant, so far as could be seen, was *S. jacobaea*. Its life-history is considered with that of *L. gracilis*.

5. *Longitarsus gracilis*, Kuts.

Two varieties of this species are recognised, var. *poweri*, All., with a black suture and var. *nigrothorax*, Hktgr., with a blackish brown thorax. Large forms, as Tomlin & Sharp remark, may be mistaken for small *L. jacobaeae*.

Food-plants.

* Heikertinger (1926) gives as its only host-plant *Tussilago farfara* and says that it occurs in fairly moist (halbfeuchtig) places. Fowler, however, records it from *S. jacobaea*, and it was on this plant that it was found in some numbers by the present writer.

Life-histories of L. succineus and L. gracilis.

Heikertinger (l.c.) gives the time of appearance and ovipositional periods of both these beetles as more or less coincident—namely in autumn, from September to mid-October.

In the "Brecklands" the adults are found much earlier than this, and already by the end of June oviposition was in progress. The eggs differ in colour, those of *L. succineus* being greenish brown, those of *gracilis* yellowish brown. They are of similar dimensions to those of *L. dorsalis*. The incubation period is from two to three weeks, and eggs were already hatching by the beginning of August. In the field, beetles

were difficult to find in October, but in the laboratory gravid females were still alive. At the same time half-grown larvae were present in the soil both in the field and in experimental pots. It is thus probable that hibernation takes place in the larval and pupal stages, but whether also in the adult stage is not clear.

Developmental Stages.

Unfortunately, owing to a mixed infection in the breeding pots, it is not possible to ascribe with certainty to one species or the other the two types of larvae obtained. In general form both resemble the species already described, and also in the arrangement of the setae on the head, thorax and abdominal segments I–VIII.

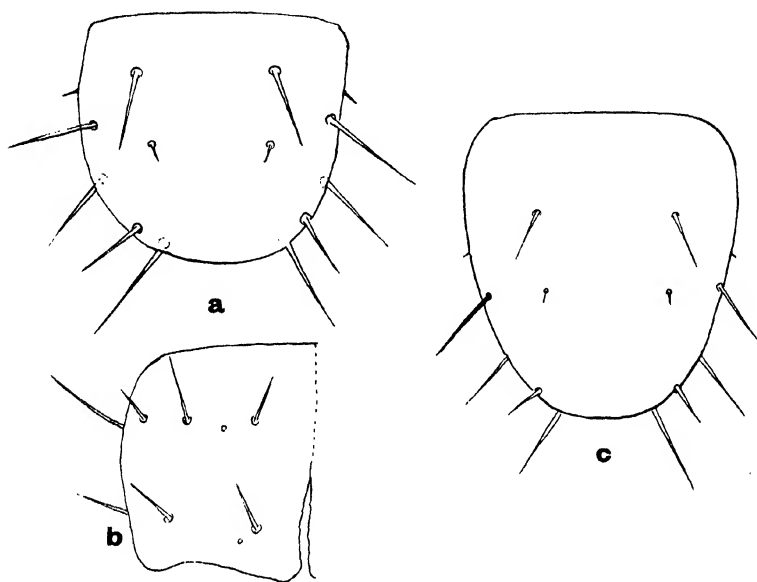


Fig. 11. a, b, anal plate and prothoracic shield of *L. succineus*, Foudr. : c, anal plate of *L. gracilis*, Kuts.

The prothoracic shield and anal plate of the newly hatched larvae probably referable to *L. succineus* is shown in fig. 11, a, b; the more elongate anal plate of *L. gracilis* in fig. 11, c.

The pupae are also similar to those of *L. jacobaeae*.

6. Effect on Ragwort.

No experiments have been made with regard to estimating the controlling influence exerted by the beetles on the spread of their host-plant. General observation, however, indicates that this effect is very small. This is largely due to the fact that the time of appearance of the new generations of the three most abundant species does not coincide with that of the germination of the seed and early stages of the seedlings. At this time the beetles are in the larval stage. These larvae will be for the most part on well established second-year plants (for *S. jacobaeae* is not an annual) and although very numerous do not noticeably affect the flowering and seeding of the plant; nor does the above-ground feeding habits of the adult beetles seriously affect the plants.

References.

- BEDEL, L. 1897-1901. Faune du Bassin de la Seine, tome v.
FOWLER, W. 1887. British Coleoptera, vol. iv. (Supplementary vol. 1913.)
HEIKERTINGER, F. 1926. Resultate fünfzehnjähriger Untersuchungen über die Nahrungspflanzen einheimischer Halticinen.—Ent. Blätt., xxii, pp. 1-9.
TOMLIN, J. R. le B. & SHARP, W. E. 1911-12. Notes on the British Species of *Longitarsus* Latr.—Ent. Mon. Mag., xlvii, pp. 274-275; xlviii, pp. 201, 252-3, 281-2.

ON THE BIOLOGY OF *PSYLLIODES HYOSCYAMI* LINN. (CHRYSOMELIDAE, COLEOPTERA), THE HENBANE FLEA-BEETLE, WITH DESCRIPTIONS OF THE LARVAL STAGES

By H. C. F. NEWTON, B.Sc., A.R.C.S.

(Entomology Department, Rothamsted Experimental
Station, Harpenden, Herts.)

(With Plates VIII and IX and 3 Text-figures.)

CONTENTS.

	PAGE
I. Introduction	153
II. The adult insect	154
III. Previous records and host plants	154
IV. Life cycle and habits	155
V. Associated insects	156
VI. Damage	156
VII. Control	157
VIII. Description of the developmental stages	157
IX. Summary	160
References	160

I. INTRODUCTION.

DURING the early summer of 1929 some specimens of Henbane (*Hyoscyamus niger* L.) were sent to this Station by Mr K. R. Reynolds from the Medicinal Herb Farm of Messrs Ransom and Son, Ltd., Hitchin. The plants were suffering from root damage, which so far as could be seen was due to millipedes and advice was given accordingly. The affected plants were kept and after a few days were noticed, in addition, to show signs of infection with numbers of Coleopterous larvae. These mined the pith, leaf stalks and leaves, and on breeding out were found to be those of *Psylliodes hyoscyami* Linn. This beetle, according to Fowler, is very rare, as a rule, and since the infestation appeared to be very heavy the farm was visited three times during 1930 and occasionally since.

The observations made together with a description of the immature stages are embodied in this note.

154 *On the Biology of Psylliodes hyoscyami Linn.*

I should like here to thank Messrs Ransom and Mr Reynolds who gave me every facility for the work and Miss A. M. Lysaght for drawing Plate VIII, fig. 1.

II. THE ADULT INSECT (Plate VIII, fig. 1).

Like the well-known "Turnip Flea," this beetle belongs to the Halticinae, a sub-section of a family of leaf-eating beetles, the Chrysomelidae. All the members of this sub-family are distinguished by the greater or less development of enlarged and muscular hind femora, conferring on them the powers of leaping which has earned them their common name of flea-beetles. The genus, *Psylliodes*, differs from all other genera by the possession of 10-jointed instead of 11-jointed antennae. The particular species is one of the larger examples and this, together with its colour, easily distinguishes it from other Halticinae feeding on Solanaceous plants.

Fowler describes it as "oblong-ovate of a bronze green or slightly coppery colour; under side nigro-aeneous; antennae black with base reddish yellow; legs reddish yellow with posterior femur dark; length 2-4 mm." Heikertinger (1912) gives a bronzy green to bronzy brown coloration as the usual form, the var. *cupronitens* Foerst as the coppery form, and var. *coerulescens* Wse as the rare green-blue form.

Of the many specimens handled in the present case there were about equal numbers of the bronze green and the blue green variations; coppery specimens were very rare.

III. PREVIOUS RECORDS AND HOST PLANTS.

Hitherto there appear to be three recent records of *Psylliodes hyoscyami* attacking medicinal crops such as henbane. Zacher (1921) records the beetle as attacking henbane in Bohemia in company with *Epitrix atropae* Foudr. and *Psylliodes affinis*, the two last named also attacking *Atropa Belladonna*. In the Crimea, Perfentjev (1921) states that though it is occasionally found on belladonna, *Psylliodes hyoscyami* more frequently attacks henbane. Van Emden (1924), in a survey of insects attacking medicinal plants in Saxony, also gives belladonna as a host plant in addition to henbane. Heikertinger (1925) considers this *Psylliodes* to be confined to *Hyoscyamus niger* in the neighbourhood of Vienna, but thinks that farther south it might be found on other species.

In the present instance, though a strip of belladonna adjoined the heavily infested henbane, few beetles were found on it, nor did they move over when the henbane was harvested in June. In the laboratory, how-

ever, beetles would eat belladonna leaves when given no other choice; *Solanum dulcamara*, *S. tuberosum* and *Nicotiana* sp. were refused.

IV. LIFE CYCLE AND HABITS.

During the winter the beetles, unlike *Psylliodes chrysocephala*, did not appear to be active but were found hibernating in the long grass bordering the field. By the end of April, though the weather was still very cold, large numbers were found collected on the roots and stems of young *Hyoscyamus* plants which had been partially buried when the land was ploughed. These, when taken to the laboratory, began oviposition during the first week of May. The eggs (Plate VIII, fig. 3 E) appear to be laid in the soil, as a search in the field showed none on the leaves or stem. That the soil is the normal place of oviposition is indicated by laboratory experiments with potted henbane plants. At the same time the surface of the *Hyoscyamus* is very hairy and covered with a sticky secretion. It is difficult to believe that the young larvae can travel over such a surface which so evidently entraps small Diptera. In any case the cast skins of first instar larvae have been found in the mines in upper regions of the plant. The eggs hatch in about a fortnight (June 14th–28th).

The whole of the larval life is passed within the plant (Plate IX, figs. 4, 5), the leaf-stalks of which are mined almost to the leaf tip, and in cases of overcrowding mines in the blade of the leaf are formed. The central pith of the main stem is also invaded and some larvae were found in the tap root. In bad infestations the stem is completely hollowed out.

Egg laying continues during May and June. By the second week of June, 1930, the oldest larvae were full grown and left the plant to enter the soil where the pupal and prepupal stages are passed (Plate VIII, fig. 3 R, P). The prepupa constructs the usual earthen cell, lining the inside surface with a cementing secretion. The majority of the pupae lie between 2 and 3 in. deep. The first were seen on June 21st and the first of the new generation emerged at the end of July; the maximum numbers a few days afterwards.

There appears to be only one generation a year and a number of the new generation were dissected at various times during the summer and early autumn, but no ripe ova were found nor were any eggs laid in captivity. It is probable that oviposition does not normally occur till the following spring. In this respect, therefore, *Psylliodes hyoscyami* differs from *P. chrysocephala* the larvae of which mine the stems of Brassicae. That species oviposits during the autumn and winter so that the winter is passed both in the adult and immature stages.

V. ASSOCIATED INSECTS.

In plants which had been attacked by the beetle larvae for some time and where rotting of the pith had begun, numbers of Cyclorrhaphous dipterous larvae were found scattered throughout the length of the stem (Plate IX, fig. 6). These, on breeding out, were determined by Mr J. E. Collin as *Lonchaea flavidipennis* Zett. He expressed the opinion that they were a secondary infection and referred to a similar case reported by Mr J. E. M. Mellor of Cambridge. Mr Mellor informed me that he had obtained the larvae of the same fly from rotting stems of parsley and although *Psila rosae* could not be definitely associated as the primary parasite he believed *Lonchaea flavidipennis* to be secondary.

In addition a predaceous Coleopterous larva was present in the stems (Plate IX, fig. 7).

VI. DAMAGE.

Although the adult beetle is capable of damaging the young plant, it is the mining habit of the larvae which is the more serious. In order to appreciate the nature of the damage the method of cultivation must be briefly described.

Henbane is a biennial. Sowing usually takes place in October, though in a bad season it may be delayed till the following spring. Two leaf crops are taken from the young plants during the first year. In the following year the plants are allowed to flower, when a further crop is taken from the flower heads and finally, during June, the whole plant is cut down at ground level. It will be seen therefore that the plant is liable to attack at several periods. The small first year plants may be attacked in the spring by the over-wintering adults and this may be followed by a larval attack as the eggs laid on them hatch. The mining of the leaves and growing point of the young plants causes a serious setback and may result in their death. In the following year attack by the over-wintered adults is not so serious, but when, as in 1930, the numbers of beetles are great, much injury is again done by the larval infestation though the plants are now full grown. Individual leaves and shoots are killed, and rotting is set up. At this stage other insects and saprophytes may follow as secondary invaders. Often the plants are killed, especially when they are also suffering from the virus diseases described by Hamilton (1932). Fortunately it appears from the experiments of this worker that the flea-beetle is not capable of transmitting the disease—at any rate in the adult stage.

VII. CONTROL.

The year during which most of these observations were made (1930) was apparently an epidemic year for many flea-beetles and the attack may have been exceptionally severe. However, where both host plant and pest are localised in distribution, eradication of the pest should be possible cheaply and easily by merely manipulating the crop.

The following are the chief points of the case under consideration. First, three areas of land are used for growing the crop, two of them adjacent—this is the infested area—the third about a mile distant. Of these three plots, one always carries first year plants, one second year plants while the third rests under a rotation crop. The two adjacent areas are in the same field, so that some henbane is always present there. Incidentally, once in three years, old and young plants are growing side by side, and this means that when the old crop is cut the beetles can migrate to the young crop. Secondly, part of the second year crop is usually left for seed, thus providing a lengthened breeding ground (for the early harvesting of the main crop in June ensures a certain measure of control as many larvae are then still within the plant). The third point is that the beetles, so far, are not present on the distant plot.

Control measures are therefore being directed towards breaking the sequence of crops on the adjacent areas, accompanied by harvesting as early and rapidly as possible and the provision made for seed production elsewhere than on the affected area.

VIII. DESCRIPTION OF THE DEVELOPMENTAL STAGES.

(a) *The egg* (Plate VIII, fig. 3 E).

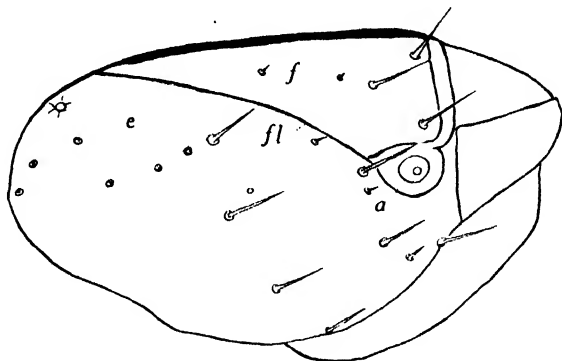
The egg is oval, $\frac{2}{3}$ mm. long by $\frac{1}{3}$ mm. in width, yellowish white in colour with a deeper yellow equatorial band in the later stages. The chorion has the fine polygonal sculpturing common to flea-beetle eggs.

(b) *The larva* (Plate VIII, fig. 2).

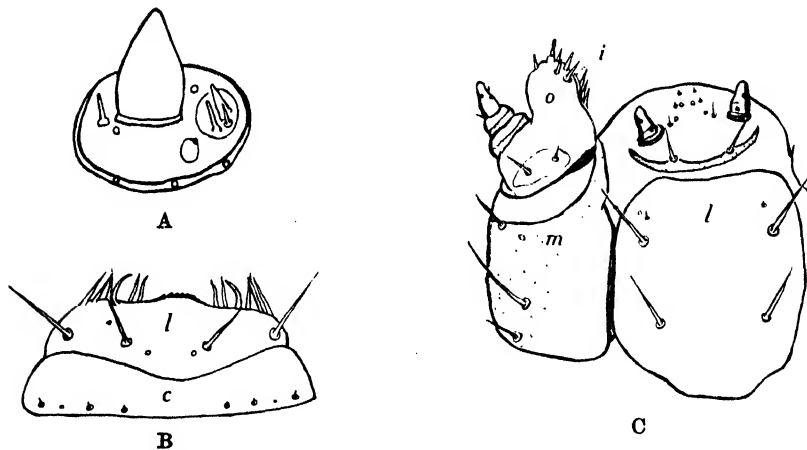
The arrangement of the chitin plates and setae of the newly hatched larva and intermediate instars is similar to that of the full-fed larva.

The full-fed larva is of the same general appearance and elongate form as other flea-beetle larvae and the following description refers chiefly to points of taxonomic value. The body colour is white with the head dark brown, prothoracic shield, anal shield and legs light brown. Average measurements are: length 8 mm., breadth 1 mm. The arrangement of the setae

on the head capsule is shown by Text-fig. 1; the antenna in Text-fig. 2 A. The strong five-toothed mandible has the usual pair of setae situated on the external face and the molar region carries four unequal sharp spines, of which the proximal is minute, projecting into the buccal cavity.

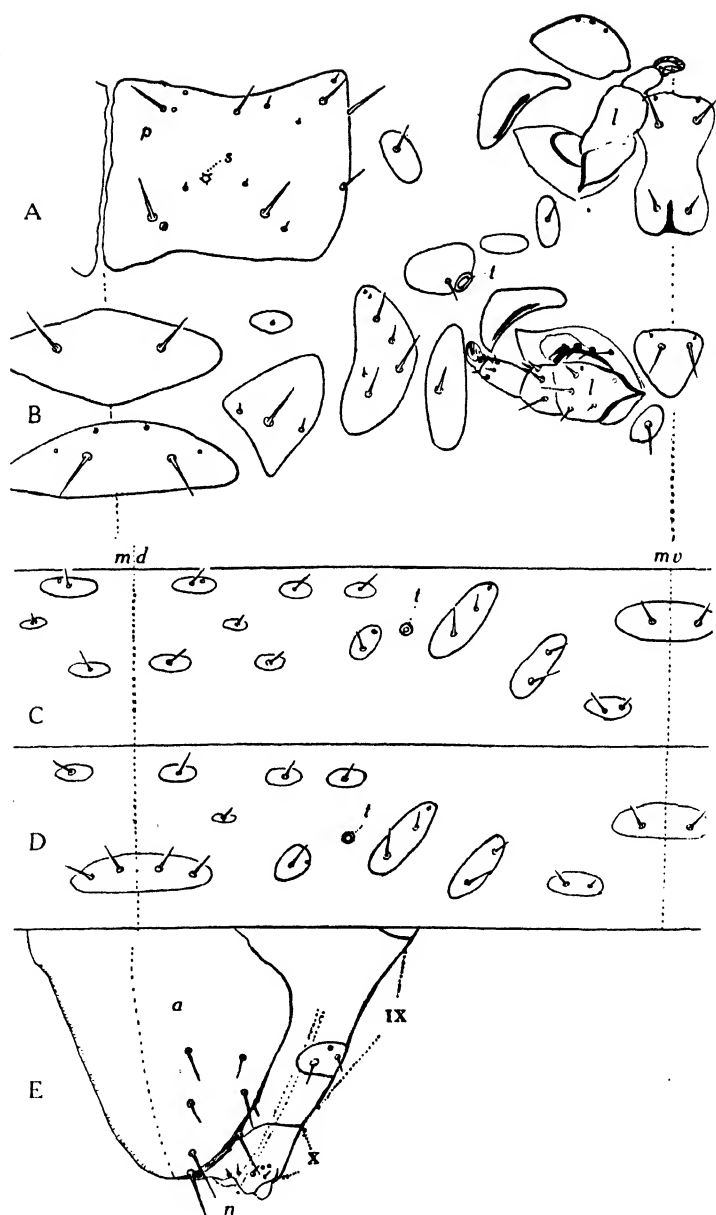


Text-fig. 1. Head capsule showing arrangement of setae. Schematic. *f*, frons; *e*, epicranium; *fl*, fronto lateral suture; *a*, antenna.



Text-fig. 2. A, left antenna and accessory sense organs. $\times 650$. B, *l* labium and *c* clypeus. $\times 170$. C, *m* maxilla and *l* labium. $\times 160$. *o* outer, *i* inner lobe. $\times 170$. Nearly full-fed larva.

Clypeus, maxilla and labium are shown in Text-figs. 2 B and C. The arrangement of the setae on the thoracic and abdominal segments is indicated by Text-fig. 3 A-E. On the prothoracic shield are two circular membranous areas (*s*) presumably sensory in function but differing from the usual form observed in flea-beetles. A pair of similar organs appears



Text-fig. 3. Arrangement of seta on the various segments. A, prothorax; B, mesothorax; C, abdominal segments 1-7; D, 8th abdominal segment; E, posterior end of larva. *p*, prothoracic shield; *s*, sense organ; *t*, spiracle; *l*, leg; *a*, anal shield; *n*, anus; IX, 9th segment; X, vestigial 10th segment; *md*, mid-dorsal; *mv*, mid-ventral line.

on the dorsal region of the epicrania of the head capsule. The metathorax is similar to the *mesothorax* except for the absence of spiracles. Abdominal segments 1-7 are similar; the 8th has the posterior dorsal plates fused, the 9th bears the anal shield, and the vestigial tenth, the anus (Text-figs. 3 C, D, E).

The pupa is of the typical flea-beetle form. It is white in colour, 4 mm. long and 2 mm. across its greater width. The chaetotaxy, which appears to be constant, is as follows. On the head three pairs of setae: on each side one below the antenna base, one at the inside margin of the eye, the third on the vertex. The prothorax bears eight pairs of setae: on each side one pair median, one antero-lateral, one median lateral, one postero-lateral and one lateral above the mesothoracic suture. Meso- and meta-thorax with four setae in a transverse line. The legs each bear two setae at the femoro-tibial bend situated externally on the tip of the femur. Abdominal segments 1-6 each with eight setae in a transverse line, the lateral seta on each side situated below the stigma on the pleura. Seventh abdominal segment shield shaped, the eight setae arranged around the posterior margin. The setae are repeated on the reduced 8th and 9th segments which are scarcely visible from above. The 9th bears the anal horns.

IX. SUMMARY.

1. A severe attack by a flea-beetle, *Psylliodes hyoscyami* Linn. on *Hyoscyamus niger* L. grown as a commercial crop is recorded.
2. The life history and developmental stages of the beetle are described.
3. Suggestions for control are made.
4. The larvae of *Lonchaea flavidipennis* Zett. (Sapromyzidae, Diptera) has been found associated with the beetle larvae in the damaged stems.

REFERENCES.

- EMDEN, FRITZ VAN (1924). Bericht über die entomologische Überwachung der Speicher und Kulturen der Firma Caesar und Loretz, A.-G. im Jahre 1923. *Jahresbericht* 1924, von Caesar und Loretz, pp. 167-232.
- FOWLER, W. W. (1887). *Coleoptera of the British Islands*, vol. IV.
- HAMILTON, MARION A. (1932). On three new virus diseases of *Hyoscyamus niger*. *Ann. Appl. Biol.* XIX, No. 4, pp. 550-67.
- HEIKERTINGER, F. (1912). In Reitter, *Fauna Germanica*, Käfer, Pt. IV.
- (1925). Resultate fünfzehnjähriger Untersuchungen über die Nahrungspflanzen einheimischer Hälticinen. *Entom. Blätt.* XXI, 81-92.
- PERFENTJEV, I. (1921). Les Insectes nuisibles aux Plantes Médicinales en Crimée. *Bull. Soc. Path. Exot. Paris*, XIV, no. 3, pp. 164-7.

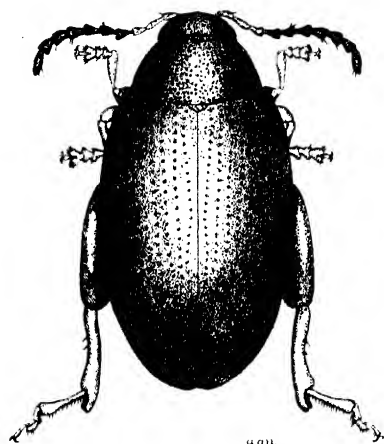


Fig. 1.



Fig. 2.



Fig. 3.

NEWTON.—ON THE BIOLOGY OF *PSYLLIODES HYOSCYAMI* LINN. (CHRYSEMELIDAE, COLEOPTERA),
THE HENBANE FLEA-BEETLE, WITH DESCRIPTIONS OF THE LARVAL STAGES (pp. 153-161).



Fig. 4.



Fig. 6.



A



B

Fig. 5.



Fig. 7.

NEWTON.—ON THE BIOLOGY OF *PSYLLIODES HYOSCYAMI* LINN. (CHRY SOMELIDAE, COLEOPTERA),
THE HENBANE FLEA-BEETLE, WITH DESCRIPTIONS OF THE LARVAL STAGES (pp. 153-161).

ZACHER, F. (1921). Tierische Schädlinge an Heil- und Giftpflanzen und ihre Bedeutung für den Arzneipflanzenbau. *Ber. deutschen Pharmazeut. Ges. Berlin*, **xxxi**, No. 2, pp. 53-65.

EXPLANATION OF PLATES VIII AND IX.

PLATE VIII.

Fig. 1. Adult beetle. $\times 15$.

Fig. 2. Full-fed larva: side, ventral, and dorsal views. $\times 4$.

Fig. 3. Eggs, E; prepupa, R; pupa, P. The pupa on the right still in its earthen cell. $\times 4$.

PLATE IX.

Fig. 4. Upper region of *Hyoscyamus* stem split to show early stages of infestation in the leaf-stalks. Larvae, L.

Fig. 5. A, later stage, leaf killed. B, larva (L), mining in leaf.

Fig. 6. Split stem of an infested plant, late stage, showing larvae of *Lonchaea flavidipennis* Zett.

Fig. 7. Predaceous Coleopterous larva from mine. $\times 25$.

(Received March 23rd, 1933.)

[Reprinted from the Journal of Agricultural Science, Vol. XXIV. Part IV.]

[All rights reserved.]

THE LOSS OF ACTIVITY OF PYRETHRUM. II.

By F. TATTERSFIELD AND J. T. MARTIN.

(*Department of Insecticides and Fungicides, Rothamsted
Experimental Station, Harpenden, Herts.*)

(With Five Text-figures.)

CONTENTS.

	PAGE
Introduction	598
Experimental	599
Exposure of pyrethrised dusts to 1000-watt lamp	599
Antioxidants and accentuation of toxicity	600
The extent to which the acid method will trace out loss of activity	602
Experiments with pyrethrised dusts	602
Comparison between two samples of pyrethrum	609
Statistical comparison of results	609
Chemical evaluation of loss of activity	615
Exposure of methyl alcoholic extract of pyrethrum	615
Exposure of pyrethrum flowers to sunlight	617
Exposure of pyrethrised dusts and pyrethrum powder in sealed flasks	619
Chemical evaluation of exposed pyrethrised dusts containing antioxidants	621
Loss of colour of pyrethrised dusts	622
Discussion	624
Summary	625
References	626

INTRODUCTION.

IN a previous paper⁽⁸⁾ of a preliminary nature it was shown that one way in which the pyrethrins lost their activity was by oxidation, particularly when exposed to light. In intense light the reaction was rapid when the pyrethrins were dispersed upon a fine inert powder so as to give a large surface area, while the incorporation of certain antioxidants tended to stabilise the pyrethrins. In the present paper the data there presented are amplified and certain corrections are made. An attempt is also made to ascertain the rate of loss of activity by exposure of dusts under more uniform conditions, to ascertain how far the acid method of estimating the pyrethrins will determine quantitatively the loss of activity under different conditions of exposure, and what degree of correlation exists between the chemical and the biological methods of evaluation used by us.

EXPERIMENTAL.

Exposure of pyrethrinised dusts to 1000-watt lamp. In the experiments previously described (*loc. cit.*), powdered pyrethrum flowers and pyrethrinised dusts were exposed to sunlight in a glasshouse. This procedure gave rise to daily variations in the amount of radiation received by the exposed samples, rendering impossible an accurate quantitative determination of the decline in activity with time. It was decided, therefore, to see how rapidly loss of toxicity would take place in pyrethrinised dusts under radiation from an artificial source of light, but not at this stage to attempt too accurate a control of the temperature. A 1000-watt tungsten filament lamp was used, together with a large white enamelled reflector, the optical contour of which was a combination of concave and cylindrical reflecting surfaces. The maximum intensity of light with even illumination was obtained in the zone of 0–45° and as far as possible the dusts were exposed within this area. The lamp operated at about 2900° K. and gave very little ultra-violet radiation. As the temperature varied from day to day, the exposure of samples to be compared was carried out in trays placed side by side at a distance of 2 ft. 3 in. from the lamp. Illumeter tests indicated the illumination at this distance to be approximately 1000 foot-candles.

The frequent stirring of the dusts was of importance, as the reaction in the presence of light was apparently a surface one. The stirring was done by hand, in the earlier experiments once every five hours, and in the later experiments every half-hour, the dusts being smoothed out as level as possible after each operation. It was found that with untreated pyrethrinised dusts the loss of activity proceeded with considerable rapidity.

In the first series of experiments, arranged to test the method, the dusts were prepared as follows: three portions of 5 gm. of a concentrated extract of pyrethrum were each dissolved in 25 c.c. of alcohol. One solution was added directly to 100 gm. of talc, and after taking off the alcohol *in vacuo* in the dark, the dried powder was finely sieved. To the other two solutions 5 gm. of hydroquinone and 5 gm. of tannic acid were added respectively, shaken till dissolved and each solution added to 100 gm. of fine talc and treated as before. Each dust contained 0.5 per cent. pyrethrin I. After thorough mixing two portions of 25 gm. of the untreated pyrethrinised dust were weighed out into flat-bottomed dishes, one of which was immediately covered with a lead sheet. 25 gm. of each of the dusts treated with antioxidant were also placed in similar

dishes. The dusts were exposed to the light of a 1000-watt lamp, in an otherwise darkened room, stirring and levelling being carried out every five hours. The maximum surface temperatures varied, and in the open dishes ranged from 29 to 38° C. but was 2° C. lower in the lead-covered dish. A sample of the untreated pyrethrised dust was kept in the dark in a cool cellar, to act as a control. It was observed that on exposure the surface yellow colour of the sample containing hydroquinone was retained with very little change, that the one containing tannic acid took on a greyish shade, whereas the colour of the uncovered untreated sample was completely discharged after a few hours. Little change was seen in the colour of the dust covered by the lead sheet. At the end of definite periods weighed quantities were taken from each dish and extracted with a known volume of alcohol. The alcoholic extracts were finally diluted with 0.5 per cent. solution of saponin in water, each dilution being brought up to the same concentration of alcohol, and sprayed upon *Aphis rumicis* in the apparatus and with the procedure previously described (9). The sprayings were carried out in triplicate at each concentration, using ten insects at a time. The results are given in Table I. The dilutions tested are expressed in terms of the content of pyrethrin I which they would have had before exposure.

The data set forth in Table I are the results of experiments carried out on three separate occasions. The effects of the sprays were judged 44 hours after their application. The data of the series *a*, *b*, *c* show that after an exposure of 42 hours the dust, shaded from the lamp by the lead sheet, is not significantly different in toxicity from the unexposed dust kept in the cellar. The sample without antioxidant shows marked degeneration after 10 hours, whereas the addition of the antioxidants, tannic acid and hydroquinone, have effected a measure of stabilisation. A slow decline in the activity of the dusts containing antioxidants does take place and the question arises, whether some of the loss of activity may not be due to reactions other than oxidation.

Antioxidants and accentuation of toxicity. In connection with the retardation of loss of activity by antioxidants one of us has previously suggested (8) that their addition to pyrethrum sprays might accentuate toxicity by preserving the activity of the pyrethrins after spraying. A carefully carried out experiment in which were compared the insecticidal values of pyrethrum extracts, with and without addition of the two antioxidants, tannic acid and hydroquinone, failed to confirm this possibility when the insects were kept in the dark after spraying. Three lots of 1.0 gm. of a concentrated extract of pyrethrum were dissolved in alcohol. To two, 1 gm. of hydroquinone and 1 gm. tannic acid were

Table I. *The effect of exposure to 1000-watt lamp upon pyrethrised talc dusts, treated and not treated with antioxidants.*Test subject, *Aphis rumicis*. M. & D.=moribund and dead, allowing for control.

Description	Concen- trations tested.	No ex- posure M. & D. %	M. & D. % after 44 hours for dusts exposed			
	Pyrethrin I in original dust					
	mg./1000 c.c.		10 hours	22 hours	32 hours	42 hours
(a) Not treated with antioxidant.	10	100	—	—	—	—
Not exposed	5	100	—	—	—	—
	2.5	92	—	—	—	—
Not treated with antioxidant.	10	—	100	100	100	100
Exposed under lead cover	5	—	100	100	100	100
	2.5	—	96	88	100	100
Not treated with antioxidant.	30	—	—	—	—	6
Exposed to 1000-watt lamp	20	—	—	—	8	0
	15	—	—	40	10	8
	10	—	84	36	0	4
	5	—	88	0	8	—
	2.5	—	52	—	—	—
Control: saponin 0.5 gm./100 c.c., alcohol 6 c.c./100 c.c.		(16.6)				
(b) Treated with tannic acid 5 %	20	—	—	—	100	—
	10	—	—	100	100	—
	7.5	100	100	100	95	—
	5	100	100	89	87.5	—
	2.5	100	86	46.5	43	—
	1	55	45	—	—	—
Control: saponin 0.5 gm./100 c.c., alcohol 6 c.c./100 c.c.		(6.6)				
(c) Treated with hydroquinone 5 %	20	—	—	—	—	100
	15	—	—	—	100	—
	10	—	—	100	100	86
	7.5	96	100	100	74	76
	5	92	88	88	53	50
	2.5	24	66	60	16	—
	1	14	40	—	—	—
Control: saponin 0.5 gm./100 c.c., alcohol 6 c.c./100 c.c.		(16.6)				

added respectively. The solutions were made up to 50 c.c. and, after dilution to the concentrations given in Table II, were tested in the usual way. Each dilution contained 0.5 gm. of saponin and 0.5 c.c. of alcohol per 100 c.c. of spray fluid. The figures given in Table II are the results of sprayings in fivefold replication, the standard error of the mean percentage of moribund and dead insects for each concentration being also given.

The data indicate that there is very little difference in the activity of the three preparations. At the concentration 4 mg. per 1000 c.c. pyrethrin I there is a fairly large discrepancy between the values for the preparations using tannic acid and hydroquinone, but an analysis of the data shows that this difference between the values is not significant. From these figures it is also safe to assume that the differences

found between the toxicities of the samples in Table I are not due to any accentuation of toxicity owing to the addition of the antioxidants.

Table II. *The effect of antioxidants on the toxicity of pyrethrum extracts.*

Insect used, <i>Aphis rumicis</i> . Fivefold replication, 10 insects at a time. Original solutions contained 0.02 gm./100 c.c. pyrethrin 1. Antioxidants 2 gm./100 c.c.				
Test	Concen- tration in pyrethrin I mg./1000 c.c.	Moribund and dead % 36 hours after spraying	Standard error %	Moribund and dead % allowing for control
Control: saponin 0.5 gm./100 c.c., alcohol 0.5 c.c./100 c.c.	—	16	±6.0	0
Pyrethrum extract untreated	10	100	—	100
	8	100	—	100
	6	100	—	100
	4	90	±4.47	88
	2	51	±5.83	41.7
Control: as above but with 0.01 gm./100 c.c. tannic acid	—	12	±5.45	0
Pyrethrum extract + tannic acid	10	100	—	100
	8	98	±2.0	97.7
	6	98	±2.0	97.7
	4	92	±2.0	91
	2	48	±2.0	41
Control: as above but with 0.01 gm./100 c.c. hydroquinone	—	4	±2.45	0
Pyrethrum extract + hydroquinone	10	100	—	100
	8	96	±2.45	96
	6	94	±6.0	94
	4	80	±6.32	79
	2	44	±5.0	41.7

THE EXTENT TO WHICH THE ACID METHOD WILL TRACE OUT LOSS OF ACTIVITY.

Experiments with pyrethrinised dusts. In an earlier paper (8) the loss of activity of pyrethrum, in a powdered form, and of pyrethrinised dusts was followed by exposing them to sunlight and periodically carrying out biological tests upon bean or wheat aphids. The objections to this method are due to difficulties and inaccuracies inherent in biological trials, chiefly owing to the fact that the operator has no assurance that the resistance of the insects to the action of the poison does not change with varying meteorological conditions. The results of tests carried out at different times might not be strictly comparable. A chemical method does not suffer from this disadvantage. Tests can be carried out on samples after prolonged exposure, and comparisons made with the original sample. This is a matter of some importance as it is known that pyrethrum may suffer slow degeneration when carefully stored in the dark, as indicated by Gnadinger and Corl (5). Before, however, attempting to employ a chemical method, it is imperative to ascertain to what

extent a correlation can be obtained between the chemical and biological examinations. In other published papers we have indicated that, in general, the determination of pyrethrin I by the acid method has placed samples in their correct order of toxicity. Our present problem is a more difficult one, as a systematic error inherent in the analytical method, although small in the case of a dust rich in pyrethrins, may have a proportionately greater significance as the activity declines on exposure.

Two samples of pyrethrised talc dusts, one relatively rich and the other poor in pyrethrin content, were prepared by extracting known quantities of powdered pyrethrum with petroleum ether and incorporating the extract with finely powdered talc, evaporating off the petroleum ether *in vacuo* and finally sieving (100 mesh to the inch). The operations were carried out in the dark and the prepared dusts stored in a cool cellar. Just before exposure the samples were analysed by the acid method⁽¹⁰⁾ with the introduction of one modification. The water-soluble pyrethrin II acid was extracted with three successive portions, of 50 c.c. each, of sodium-treated ether in a separating funnel, each ether extract being washed with a little distilled water. Portions of 20 gm. of the samples were spread evenly in a thin layer in circular trays of the same diameter and exposed in symmetrically disposed positions under a 1000-watt lamp, the distance from the lamp being the same in each case. Every half-hour the samples were stirred and again evenly spread. The samples were taken off at the end of the 2, 4, 8, 12, 16 and 60 hours' exposure in the case of the poor dust, and at the end of 4, 8, 12, 16, 20 and 50 hours' exposure in the case of the rich dust. An aliquot part of each was analysed and the remainder of the sample stored for a short time in a closed container in a cool cellar and used for biological tests. The results of the analyses are given both in tabular and graphical form later in this paper (Table VII, Fig. 3).

In the majority of the biological trials, for each concentration tested, fifty insects were used, and these were selected from the adult apterous females in order to obviate difficulties arising from ecdysis in modifying resistance.

In general, only two or at the most three samples were tested on the same day and strict comparisons made only for those samples, but a preliminary series of tests was carried out on five samples of the poorer dusts which included the unexposed, and those exposed 4, 8, 12 and 16 hours. The tests were only made in duplicate and therefore strictly quantitative deductions cannot be drawn as to the relative activities of the samples. The relevant data are given in Table III.

Table III. *Toxicity of extracts prepared from dusts before and after exposure to 1000-watt lamp.*

Insect used, *Aphis rumicis*. Tests in duplicate, 10 insects at a time.
 Examination 2-3 days after spraying. M. & D.=moribund and dead insects.
 Concentrations mg./1000 c.c. tested
 in terms of

Description of test	<div> <div>Pyrethrin I before exposure</div> <div>Total pyrethrins before exposure</div> <div>Pyrethrin I after exposure</div> <div>Total pyrethrins after exposure</div> </div>				M. & D. %
	Pyrethrin I before exposure	Total pyrethrins before exposure	Pyrethrin I after exposure	Total pyrethrins after exposure	
Pyrethrinised talc dust	11	24	—	—	100
Not exposed	9	19	—	—	80-100
Analysis:	7	14	—	—	80-100
pyrethrin I 0.26 %	4.5	9.5	—	—	52-62
pyrethrin II 0.29 %	2	5	—	—	10-35
Exposed 4 hours	34	72	29	64	100
Analysis:	27	57	23	50	100
pyrethrin I 0.22 %	18	38	15	34	77-85
pyrethrin II 0.27 %	14	29	11	25	75-90
	9	19	7.5	17	45-57
	4.5	9.5	4	8	21-42
Exposed 8 hours	90	90	18	110	100
Analysis:	70	145	13	83	100
pyrethrin I 0.05 %	45	95	8.7	56	100
pyrethrin II 0.27 %	35	70	6.5	41	82
	20	50	4	25	67-71
	10	24	2	12	15-35
Exposed 12 hours	680	1440	60	610	100
Analysis:	450	950	40	405	100
pyrethrin I 0.024 %	220	470	20	200	100
pyrethrin II 0.22 %	110	240	10	100	72-95
	56	120	5	50	40-70
	23	50	2	20	21-26
Exposed 16 hours	1360	2860	104	985	100
Analysis:	900	1900	70	665	100
pyrethrin I 0.02 %	680	1440	50	470	100
pyrethrin II 0.17 %	450	950	35	330	80-95
	230	480	17	165	50-65
Control:					2.5-17.5
0.5 gm./100 c.c. saponin					
and 5.0 c.c./100 c.c. alcohol					

The figures in the table show that although toxicity rapidly falls off after the 4-hour period, some activity persists after 16 hours' exposure, but the toxicity is apparently not commensurate with the small residual value of matter expressed as pyrethrin I (0.02 per cent.) which is probably extraneous matter. On the whole, as far as one can judge from these data, the estimation of pyrethrin I traces out the loss of activity better than the figures expressed as total pyrethrins, but some correction for an error in the estimation of pyrethrin I is required. As in this series too few insects were used for the employment of statistical analysis, a second

series of insecticidal tests was carried out in fivefold replication, in which the samples of unexposed dust and those exposed to a 1000-watt lamp for 8 and 16 hours were used. The subsequent operations were carried out as far as possible in the dark. After extraction with petroleum ether in the dark the solvent was taken off in a stream of carbon dioxide and finally *in vacuo*, the residue was extracted, with cooling, with five lots of methyl alcohol, which was finally taken off *in vacuo*, and the residue dissolved in a small amount of absolute alcohol and made up to a known volume with 0.5 per cent. saponin solution. The alcohol content for each dilution was adjusted to 5 c.c. per 100 c.c. of solution. The insects were sprayed in a modification of the apparatus previously employed, an account of which will be subsequently published. Owing to the difficulty encountered in keeping the foliage, near which the insects were placed, fresh in petri dishes during the hot dry weather, the insects after spraying were placed without handling in tubes afterwards covered by muslin and kept in a cool cellar. Examination of the insects was made 20 and again 44 hours after spraying. The data are set out in Table IV and expressed graphically in Fig. 1, section A.

Table IV. *Insecticidal tests with pyrethrinised talc dusts exposed for different periods to 1000-watt lamp.*

Insect used, *Aphis rumicis*. Tests in fivefold replication, 10 insects at a time. Results 44 hours after spraying. The dust exposed was poor in pyrethrin content. M. & D. = moribund and dead.

Concentrations mg./1000 c.c., tested,
in terms of

Description	Pyrethrin I as found	Pyrethrin II as found	Total pyrethrins as found	Pyrethrin I adjusted	Total pyrethrins with adjusted pyrethrin I	M. & D. after 44 hours %	Standard error %	M. & D. allowing for control %	Probits (Bliss)
Unexposed	11.3	12.5	24	10	22.5	100	—	100	—
Analysis:	9	10	19	8.3	18	100	—	100	—
pyrethrin I 0.26 %	6.8	7.5	14	6.3	14	100	—	100	7.722
pyrethrin II 0.27 %	4.5	5	9.5	4.2	9	92	±3.7	91.8	6.392
pyrethrin I after deducting 0.02 % = 0.24 %	2.3	2.5	5	2.1	4.5	40	±4.47	38.8	4.715
	1.1	1.2	2	1	2	4	±2.4	2.04	2.954
Exposed 8 hours	7.2	35	42	4.6	39.5	78?	±9.7	77.5?	—
Analysis:	6.0	29	35	3.6	32.5	76	±9.3	75.5	5.680
pyrethrin I 0.055 %	4.8	23	28	3.05	26	68	±6.6	67.3	5.448
pyrethrin II 0.25 %	4.2	20.5	24.5	2.65	23	58	±8.0	57.1	5.179
pyrethrin I after deducting 0.02 % = 0.035 %	3.6	17.5	21	2.3	20	55	±5.0	54.0	5.100
	3.0	14.5	17.5	1.9	16.5	38	±3.7	36.7	4.060
	2.4	11.5	14	1.5	13	8	±4.9	6.1	3.454
Exposed 16 hours	35	280	315	—	—	92.8	±3.7	92.6	—
Analysis:	26	210	236	—	—	70	±7.0	69.4	—
pyrethrin I 0.02 %	17	140	157	—	—	70.4	±7.3	69.8	—
pyrethrin II 0.16 %	8.7	70	79	—	—	34	±4.0	32.6	—
pyrethrin I after deducting 0.02 % = nil	4.3	35	39	—	—	4	±3.0	2.04	—
Control:						2			
saponin 0.5 gm./100 c.c. + alcohol 5 c.c./100 c.c.									

The figures for pyrethrin I and II, found by the determination of the volatile and water-soluble acids, are shown in columns 2 and 3 of the table. The amount of pyrethrin I steadily declined, the value for the

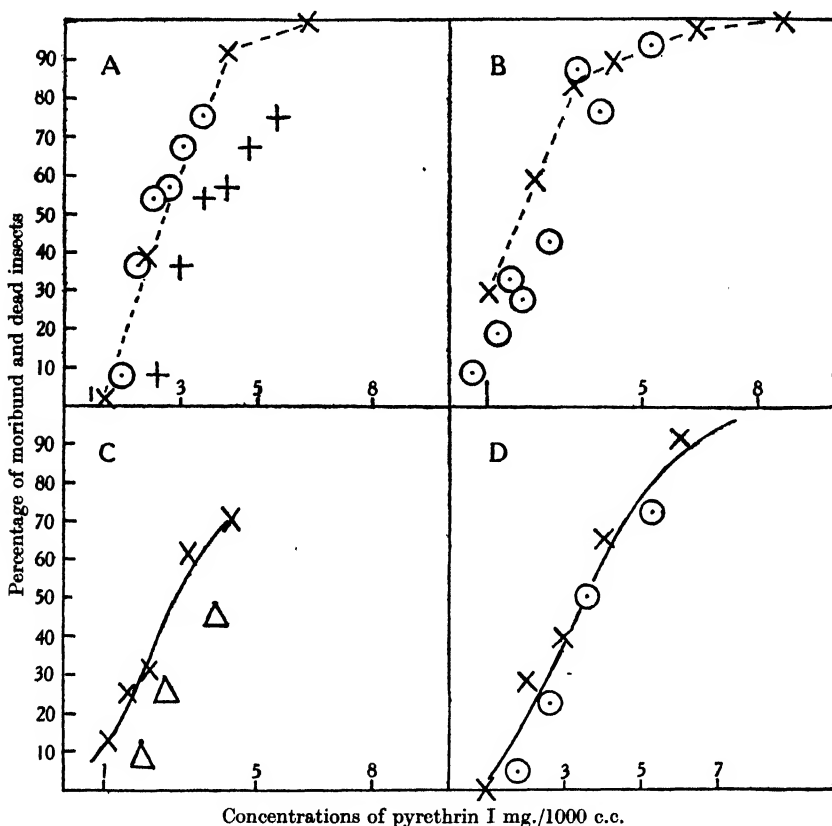


Fig. 1. Relation between concentration of pyrethrin I and toxicity. Pyrethrinised dusts exposed for different periods.

Section A. Poor dust.

- x Unexposed (corrected).
- Exposed 8 hours (corrected).
- + Exposed 8 hours (uncorrected).

Section B. Rich dust.

- x Unexposed (corrected).
- Exposed 8 hours (corrected).

Section C. Rich dust.

- x Unexposed (corrected).
- △ Exposed 6 hours (corrected).

Section D.

- x Harpenden flowers 1932 (corrected).
- Dalmatian flowers No. 1606 (corrected).

sample exposed 16 hours being very small, whereas the pyrethrin II value after 16 hours' exposure is still appreciable in amount. An examination of the corresponding toxicities of the three samples shows that the toxicity values for the sample exposed 16 hours are not com-

mensurate with even the small residual value expressed as pyrethrin I, and the conclusion is drawn that the residue of 0.02 per cent. is probably non-pyrethrin matter. We have, therefore, felt justified in deducting this amount from the values of the other two samples, so that the figures in column 5 representing the concentrations tested in the case of the samples labelled "unexposed" and "exposed 8 hours" are corrected for this residue. A comparison between columns 2 and 5 will show that the correction in the case of the unexposed samples has but a small effect upon the concentrations tested, but that in the case of the sample exposed 8 hours it is of greater significance. The percentages of moribund and dead insects in columns 7 and 9, for the sample exposed 8 hours, are in much closer agreement with those given by the unexposed samples when the adjusted figures are taken.

In section A of Fig. 1 the results corrected for an error of 0.02 per cent. pyrethrin I are plotted, but the uncorrected values are also shown (by points marked +). It is obvious that in the latter case there is a departure from coincidence.

Two further series of tests were carried out on a talc dust rich in pyrethrins, the results of which are given in the series A and B of Table V.

In series A of Table V, the data afford a comparison for three samples: (1) the unexposed, (2) exposed 8 hours, (3) exposed 16 hours. An exposure of 8 hours has led to a big reduction in the amount of pyrethrin I and a further exposure of 8 hours has led to a further reduction to 0.044 per cent. Known quantities of exposed and non-exposed samples were extracted and diluted as previously described. The spray tests were carried out in fivefold replication and the percentage number of moribund and dead insects determined after about 20 and again after 44 hours. The values obtained after the latter interval are expressed in the table, both with and without a correction for the control sprays, for which a saponin and alcohol solution of the same strength as that used in the dilutions was tested. The data given indicate that the dilutions from the extract of the sample exposed 16 hours do not give results commensurate with the small residue (0.044 per cent.) expressed as pyrethrin I, and the conclusion is drawn, as in the previous case, that the residue is non-pyrethrin material, and that its deduction from the values found for the samples, unexposed and exposed 8 hours, would probably give a better evaluation of those samples. The corrected values for pyrethrin I are given in column 5. The concentrations thus corrected are plotted against the percentage number of moribund and dead insects in Fig. 1, section B.

Table V. *Insecticide tests with pyrethrinised talc dusts exposed for different periods to 1000-watt lamp.*

Insect used, *Aphis rumicis*. Tests in fivefold replication, 10 insects at a time. Results 44 hours after spraying. The dust exposed was rich in pyrethrins. M. & D. = moribund and dead.

Concentrations mg./1000 c.c., tested,
in terms of

Description	Pyrethrin I as found	Pyrethrin II as found	Total pyrethrins as found	Pyrethrin I adjusted	Total pyrethrins with adjusted pyrethrin I	M. & D. after 44 hours %	Standard error %	M. & D. allowing for control %	Probits (Bliss)
Series A*									
Unexposed	9.1	8.3	17.4	8.6	16.9	100	—	100	7.854
Analysis:	6.9	6.2	13.1	6.4	12.6	98	±2	97.8	7.015
pyrethrin I 0.698 %	4.6	4.1	8.7	4.2	8.3	90	±1	89.3	6.243
pyrethrin II 0.63 %	3.4	3.0	6.4	3.2	6.2	84.4	±1.1	83.4	5.970
pyrethrin I after deducting 0.04 % = 0.06 %	2.3	2.1	4.4	2.2	4.3	61.2	±1.4	58.6	5.217
	1.1	1.0	2.1	1.0	2.0	34	±5.1	29.6	4.464†
Exposed 8 hours	7.9	23.0	31	5.2	28	94	±4	93.6	6.522
Analysis:	5.9	17.0	23	3.9	21	78	±5.8	76.5	5.723
pyrethrin I 0.12 %	4.9	14.0	19	3.3	17	88	±5.8	87.2	6.136
pyrethrin II 0.34 %	3.9	11.0	15	2.6	13.6	46†	±11.7	42.4†	4.808†
pyrethrin I after deducting 0.04 % = 0.06 %	2.9	8.5	11	1.9	10	32	±5.8	27.5	4.402
	2.46	7.0	9.5	1.6	8.6	37.2	±6.6	33	4.560
	1.96	5.7	8	1.3	7.3	24	±4	18.9	4.118
	1.0	3.0	4	0.65	3.6	14.3	±5.1	8.6	3.634†
Exposed 16 hours	28.6	157	186	—	—	82.3	±7.4	81.1	—
Analysis:	21.6	118	140	—	—	80	±4.5	78.7	—
pyrethrin I 0.044 %	14.4	78	92	—	—	66.6†	±9.4	64.4	—
pyrethrin II 0.23 %	7.0	39	46	—	—	52	±3.7	48.8	—
pyrethrin I after deducting 0.04 % = 0 %	3.5	19	23	—	—	23.4†	±8.8	18.3	—
Control:						6.25	±6		
saponin 0.5 gm./100 c.c. + alcohol 5 c.c./100 c.c.									
Series B*									
Unexposed	4.6	4.1	8.7	4.3	8.4	72	±10.7	70.8	5.548
Analysis:	3.4	3.0	6.4	3.2	6.2	63.2	±7.0	61.6	5.295
pyrethrin I 0.698 %	2.3	2.1	4.4	2.2	4.3	34	±6.0	31.2	4.510
pyrethrin II 0.63 %	1.7	1.5	3.2	1.6	3.1	28	±5.8	25	4.325†
pyrethrin I after deducting 0.04 % = 0.06 %	1.1	1.0	2.1	1.07	2.1	16.6	±5.3	13.1	3.878†
Exposed 6 hours	4.9	11.4	16	3.9	15	50	±7.1	47.9	4.947
Analysis:	3.3	7.7	11	2.6	10	32	±3.7	29.2	4.452
pyrethrin I 0.2 %	2.5	5.8	8	2.0	8	16	±7.5	12.5	3.850
pyrethrin II 0.46 %	1.6	3.8	5	1.3	5	4	±2.45	0	—
pyrethrin I after deducting 0.04 % = 0.16 %	0.8	1.4	2	0.65	2	4	±2.45	0	—
Controls:						4.2	±4.0		
saponin 0.5 gm./100 c.c. + alcohol 5 c.c./100 c.c.						4.0	±2.45		

* Results in series A and B cannot be strictly compared as the tests were carried out on separate days.

† Omitted in calculating regression line.

‡ Tests in triplicate.

Section B of Table V gives the insecticidal results for the samples unexposed and exposed 6 hours. An attempt was made to explore the region of the 50 per cent. mortality, but the data obtained were not complete, as with the sample exposed 6 hours the two lowest concen-

trations gave results not differing from those given by the controls, and the mortalities did not extend above the 50 per cent. death point.

Again an allowance of 0.04 per cent. in the estimation of pyrethrin I gave the closer correlation between concentration and effect. In Fig. 1, section C, this corrected concentration is plotted against percentage number of moribund and dead insects.

It was not expected that the estimation of pyrethrin I would evaluate completely the samples throughout the whole range of activity during exposure. In addition to the part played by non-pyrethrin compounds, the action of pyrethrin II is bound to have some effect, and it is a matter for some regret that our data cannot be regarded as demonstrating its extent. We are not satisfied that the acid method will show up the loss of pyrethrin II with precision, and we consider further work will be required on this point. The investigations outlined in the foregoing section indicate that, with a correction which is probably only just outside experimental error, the determination of pyrethrin I in the case of normal samples enables us to follow approximately the loss of activity.

Comparison between two samples of pyrethrum. In order to compare the results given by the acid method of analysis with those of biological trials, flowers grown in Harpenden and rich in pyrethrins and a Dalmatian sample poor in pyrethrins were analysed. The Harpenden sample was of the previous year's crop, whereas the Dalmatian sample was probably some years old. After the analysis, known amounts of each were extracted and diluted as before. Dilutions were made with 0.5 per cent. saponin and brought up to the same percentage content of alcohol. The results are given in Table VI.

It was found that the Harpenden sample after prolonged exposure gave a residual value of 0.036 per cent. of apparent pyrethrin I. A value of 0.04 per cent. was deducted from the estimates of pyrethrin I and the dilutions, adjusted for this value, are expressed in column 5. Corrected concentrations are plotted against the percentage number of moribund and dead insects in section D of Fig. 1.

It may be pointed out that the values of the concentrations of the extracts adjusted for an error of 0.04 per cent. gave results in closer agreement than the uncorrected values, and it is obvious that in Fig. 1, section D, one curve could be drawn which would fit both sets of data quite closely.

Statistical comparison of results. The statistical comparison of different experimental series relating the kill of aphides to the concentration of the pyrethrins is not an easy matter, since curves of this type commonly

Table VI. *Insecticide tests with two samples of pyrethrum, Harpenden 1932 crop and Dalmatian No. 1606.*

Insect used, *Aphis rumicis*. Tests in fivefold replication, 10 insects at a time.
Results 44 hours after spraying. M. & D. = moribund and dead.

Concentrations mg./1000 c.c., tested,
in terms of

Description	Concentrations mg./1000 c.c., tested, in terms of					M. & D. after 44 hours	Standard error %	M. & D. allowing for control %	Probits (Bliss)
	Pyrethrin I as found	Pyrethrin II as found	Total pyrethrins as found	Pyrethrin I adjusted	Total pyrethrins with adjusted pyrethrin I				
Harpenden	6.4	7.2	13.6	6.0	13.2	92	±5.8	91.3	6.380
Analysis:	4.3	4.8	9.1	4.0	8.8	68	±7.3	65.2	5.391
pyrethrin I 0.63 %	3.2	3.6	6.8	3.0	6.6	44.9	±4.4	40.1	4.749
pyrethrin II 0.71 %	2.1	2.4	4.5	2.0	4.4	34	±3.6	28.3	4.426*
pyrethrin I after deducting 0.04 % = 0.59 %	1.1	1.2	2.3	1.0	2.2	8	±3.7	0	—
Dalmatian 1606	6.7	8.8	15.5	5.3	14.0	74	±6.0	71.7	5.574
Analysis:	4.5	5.9	10.4	3.5	9.4	54	±6.0	50	5.000
pyrethrin I 0.19 %	3.35	4.4	7.7	2.65	7.0	28.5	±3.9	22.3	4.238
pyrethrin II 0.25 %	2.2	2.9	5.1	1.76	4.7	12	±2.0	4.3	3.283
pyrethrin I after deducting 0.04 % = 0.15 %	1.1	1.5	2.6	0.88	2.4	2	±2.0	—	—
Control:						8	±2.0		
saponin 0.5 gm./100 c.c.									
+ alcohol 5 c.c./100 c.c.									

* Omitted in calculating regression line.

are sigmoid in character. Recently, however, it has been shown by Hemmingsen⁽⁶⁾ and Gaddum⁽³⁾ and by Bliss⁽¹⁾, working with different organisms and poisons, that if the dosage is expressed in logarithms, the resulting sigmoid curves are symmetrical, may be identified as cumulative normal frequency distributions, representing the variation between individuals in their susceptibility to the toxic agent, and can be transformed to straight lines. Their method is based on the following reasoning. Assuming that the minimum lethal dose of a poison is an index to susceptibility, if this could be determined exactly for each individual, the distribution of the number of individuals having each degree of susceptibility might be expected to follow the normal curve of error, which has been found to hold so widely elsewhere in biology. Actually, any given concentration, applied to a random sample of organisms, will kill not only those individuals requiring this particular minimum lethal dose but also all more susceptible individuals, so that instead of a differential curve of distribution, a series of concentrations will give an S-shaped cumulative or integral curve. In this curve each percentage kill corresponds to the area under the differential curve from the dosage (susceptibility) giving the smallest lethal effect up to a perpendicular erected at the dosage actually administered. By means of tables of the normal curve the expected dosage (susceptibility) expressed in units

of the standard deviation may be read directly from the area represented by each percentage kill. If this "expected" dosage is plotted against the logarithm of the administered dosage the points will fall into a straight line. By means of this transformation to a straight line the relation between mortality and dosage can be computed for any given series within known limits of accuracy and the agreement or disagreement of two or more series determined. Our procedure has been to show, first, whether these dosage-mortality curves, transformed as above, differed significantly from parallelism and, secondly, whether they differed significantly from coincidence.

In carrying out the insecticidal tests, we attempted to explore the neighbourhood of the concentrations giving 50 per cent. moribund and dead, as we believe that here the most accurate comparisons can be made. We were not entirely successful. McCallan and Wilcoxon⁽⁷⁾, who have examined the form of the toxicity surfaces for copper sulphate and sulphur, state that the 50 per cent. mortality point may not be the most accurate for purposes of comparison; nevertheless, the extremes of the curve should be avoided, and this seems to be a conclusion common to many investigators. The advantage derived from the use of the device outlined above is that one is enabled by its means to utilise a large range of data. We originally tested our data by means of Bliss's probits as first published⁽¹⁾, but as this list has been discarded the probits from this table are not tabulated. We have finally used his more recent table which he most kindly placed at our disposal in manuscript. In the latter case the method of weighting, worked out by him and Prof. R. A. Fisher (shortly to be published), has been used. In order to incorporate tests giving no survivors the correction recently determined by Bliss and Fisher has also been used. We have found that the lowest concentrations, when the tests were least accurate, frequently gave values departing from the general trend of the results. This has been noted in many quantitative biological evaluations of this type. Such values have been neglected and occasionally we have discarded a value which from the nature of the data has been open to suspicion of grave inaccuracies; *e.g.* two values marked ? in Tables IV and V have been neglected, as there was good reason for considering that they had been seriously influenced by the imperfect sampling of the insect population. Our procedure was to plot the probit values against the logarithms of the concentrations of pyrethrin I (corrected and uncorrected) and total pyrethrins for the various sets of data in Tables IV, V, VI. The figures for the following experiments were graphed: (1) poor dust not exposed and

exposed 8 hours, (2) rich dust not exposed and exposed 8 hours, (3) rich dust not exposed and exposed 6 hours, (4) two unexposed samples of Harpenden and Dalmatian pyrethrum powder. It was observed that the corrected pyrethrin I values gave regression lines much nearer to coincidence for each of the above pairs of tests than the uncorrected pyrethrin I values or the estimations of total pyrethrins. It was, therefore, decided to ascertain the degree of concordance in the parallelism and in the coincidence of each pair of regression lines by Bliss's method. The constants of the regression lines of probits upon the logarithms of the concentrations, corrected for the residue of apparently non-pyrethrin matter, were determined, for each pair of the above tests, from the equation $Y = a + b(x - \bar{x})$. The determination of χ^2 for each line determined the goodness of fit. From the data so obtained the lines were tested for departure from parallelism and departure from coincidence by the χ^2 test as given by Bliss and by the table of "t" (2). The formulae used for the χ^2 tests were

$$\chi_b^2 = \frac{(b_1 - b_{11})^2}{\frac{1}{[\sum (wx^2) - \bar{x}\sum wx]_1} + \frac{1}{[\sum (wx^2) - \bar{x}\sum wx]_{11}}},$$

$$\chi_a^2 = \frac{[(a_1 - a_{11}) - b_c(\bar{x}_1 - \bar{x}_{11})]^2}{\frac{1}{\sum w_1} + \frac{1}{\sum w_{11}}}.$$

b_c , the mean slope of the two lines, is given by

$$b_c = \frac{[\sum wxy - \bar{x}\sum wy]_1 + [\sum wxy - \bar{x}\sum wy]_{11}}{[\sum (wx^2) - \bar{x}\sum wx]_1 + [\sum (wx^2) - \bar{x}\sum wx]_{11}},$$

where w is the weighting factor, the subscripts $_1, _{11}$ indicate one or other of the regression lines being compared, \bar{x} is the mean value of x (logs of concentrations), y are probits.

The use of table of "t" involved the calculation of

$$t_a = \frac{(a_1 - a_{11}) - b_c(\bar{x}_1 - \bar{x}_{11})}{\sqrt{s_{a_1}^2 + s_{a_{11}}^2}},$$

$$t_b = \frac{b_1 - b_{11}}{\sqrt{s_{b_1}^2 + s_{b_{11}}^2}},$$

where a_1, a_{11}, b_1, b_{11} are the constants of the regression lines, $s_{a_1}, s_{a_{11}}$ are the standard errors of the a values of the two series given by

$$s_{a_1} = \sqrt{\frac{\chi_1^2}{n_1 \sum w_1}}, \quad s_{a_{11}} = \sqrt{\frac{\chi_{11}^2}{n_{11} \sum w_{11}}},$$

s_{b_1} , $s_{b_{11}}$ are the standard errors of the b values of the two series, given by

$$s_{b_1} = \sqrt{\frac{\chi_1^2}{n_1 [\Sigma (wx^2) - \bar{x} \Sigma wx]_1}}, \quad s_{b_{11}} = \sqrt{\frac{\chi_{11}^2}{n_{11} [\Sigma (wx^2) - \bar{x} \Sigma wx]_{11}}},$$

the other symbols having the same significance as for the χ^2 test.

The χ^2 table was entered at $n=1$ and the table of " t " at n =the numbers of degrees of freedom.

The calculated weighted regression lines for the four series are plotted in Fig. 2. As a result of the statistical examinations the following conclusions were drawn:

Data of Table IV, Fig. 2, section A.

$$\chi_b^2 = 0.246, \chi_a^2 = 0.484, t_b = 0.509, t_a = 0.946.$$

Regression lines for unexposed and exposed samples do not depart significantly from parallelism or coincidence.

Data of Table V, series A, Fig. 2, section B.

$$\chi_b^2 = 0.047, \chi_a^2 = 2.561, t_b = 0.272, t_a = 1.567.$$

Regression lines for unexposed and exposed samples do not depart significantly from parallelism or coincidence.

Data of Table V, series B, Fig. 2, section C.

$$\chi_b^2 = 0.001, \chi_a^2 = 10.009, t_b = 0.122, t_a = 4.344.$$

Lines show a significant non-departure from parallelism but show a significant departure from coincidence by the χ^2 test. Departure from coincidence by the " t " test just significant¹.

Data of Table VI, Fig. 2, section D.

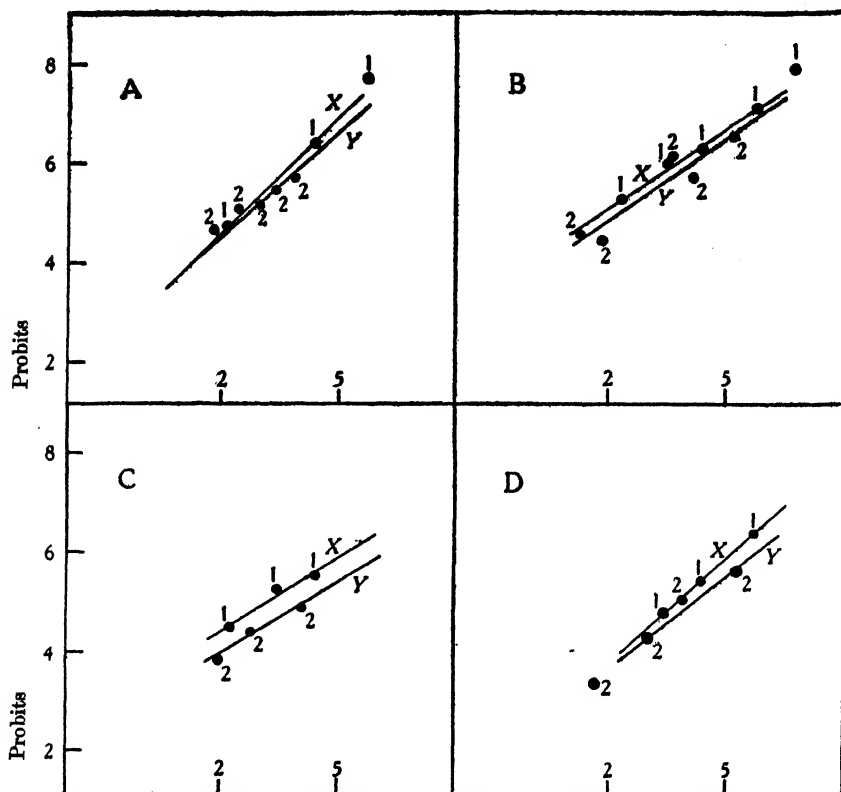
$$\chi_b^2 = 0.210, \chi_a^2 = 3.250, t_b = 1.042, t_a = 3.418.$$

Regression lines for Harpenden and Dalmatian flowers do not depart significantly from parallelism or coincidence.

It was noted in carrying out these tests that, in the first two series (Fig. 2, sections A and B) where the activity has fallen to a low level in the exposed samples, the points were more irregularly distributed about the regression lines than for the unexposed samples and that in general the regression lines for the exposed samples were slightly below those of the unexposed samples. The irregularity shown by the exposed samples

¹ Similar results were obtained on statistical examination of the whole of the data taking the results of the spraying of each batch of ten insects separately. The departure from coincidence may be due to the fact that the sample was taken when loss of activity was proceeding at a rapid rate.

may be due to the fact that, in order to obtain concentrations of pyrethrin I equivalent to those of the unexposed samples, much larger amounts of the dust had to be extracted, introducing a much higher proportion of non-pyrethrin material into the spray fluids. Thus, although the content



Concentrations of adjusted pyrethrin I mg./1000 c.c. expressed on logarithmic scale

Fig. 2.

Section A. Poor dust (data Table IV).

X Unexposed ●¹

Y Exposed 8 hours ●²

Section C. Rich dust (Table V, series B).

X Unexposed ●¹

Y Exposed 6 hours ●²

Section B. Rich dust (Table V, series A).

X Unexposed ●¹

Y Exposed 8 hours ●²

Section D. Data Table VI.

X Harpenden 1932 flowers ●¹

Y Dalmatian 1606 flowers ●²

of pyrethrin I in the whole spray fluid might be the same for equivalent dilutions for both the exposed and unexposed samples, the dilution in the dispersed phase would be greater in the case of the exposed sample. We know of no exact information on the influence of such disperse phase dilution, but it might well be important and render more difficult an

exact comparison of our samples. The conclusion that we feel entitled to draw from the above analysis is, that the estimation of the volatile acid expressed in terms of pyrethrin I and corrected for a small residual value will enable us to trace out loss of activity with approximate accuracy.

Chemical evaluation of loss of activity. Table VII gives the analytical data for the change in composition of the pyrethrised dusts used in the biological trials on exposure to a 1000-watt lamp. The results are expressed graphically in Fig. 3.

Table VII. *Analysis of dusts exposed for different periods to 1000-watt lamp.*

Dusts spread in thin layers, and stirred every half hour.

	Exposure periods in hours						
	Not exposed	4	6	8	12	16	Over 50
Low quality dust:							
Pyrethrin I %	0.26	0.22	—	0.06	0.03	0.02	0.02
Pyrethrin I % corrected	0.24	0.20	—	0.04	0.01	Nil	Nil
Pyrethrin II %	0.27	0.25	—	0.25	0.21	0.16	0.12
High quality dust:							
Pyrethrin I %	0.70	0.65	0.20	0.12	0.05	0.04	0.03
Pyrethrin I % corrected	0.66	0.61	0.16	0.08	0.01	Nil	Nil
Pyrethrin II %	0.63	0.63	0.46	0.34	0.20	0.23	0.15

The data show that there is a definite lag period at the commencement of the exposure, followed by a steep decline in the amount of pyrethrin I. The duration of the initial lag period is largely, but probably not wholly, a function of the frequency of stirring. In the above cases the dusts were stirred every half-hour, but if the periods between stirring are lengthened the lag period becomes greater, thus if a rich dust is stirred twice a day during exposure the rapid decline in pyrethrin I does not take place until after some ten hours. This is partly explicable on the grounds that the reaction is a surface one. Pyrethrin II appears to be destroyed more slowly, on the whole, than pyrethrin I, particularly towards the end of the exposure. Extracts of the dusts exposed for 16 and 50 hours have still a small residual activity.

Exposure of methyl alcoholic extract of pyrethrum. A preliminary test on the effect of the exposure to sunlight and air of a methyl alcoholic extract of pyrethrum was carried out. The solution was placed in a cylindrical glass vessel to which was attached a condenser, and a stream of air was passed through the apparatus by means of a small electric pump. Thorough aeration of the solution was brought about by passing the air through a sintered glass plate at the bottom of the vessel. The

apparatus was exposed to bright sunlight in a warm glasshouse. The radiation figures on the days of exposure were of a constant high level. The temperature throughout the experimental period approximated closely to 25° C.

The solution was prepared for exposure by the extraction of Harpenden grown flowers (1932 crop) with petroleum ether, removal of the solvent, the residue being then extracted with five portions of 20 c.c. of warm methyl alcohol. Each alcoholic extract was cooled, and filtered.

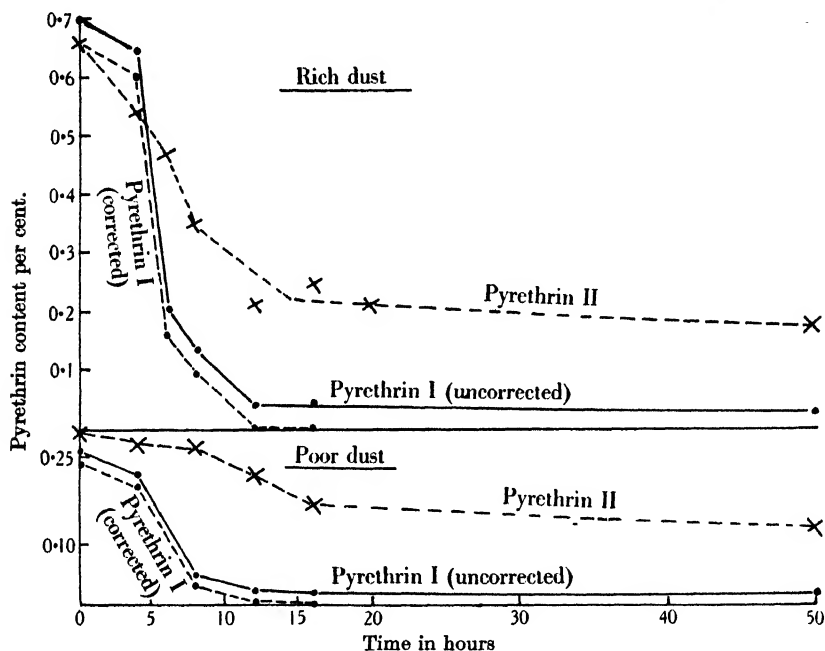


Fig. 3. Decline in pyrethrin values on exposure of rich and poor dusts.

The combined filtrates were adjusted to a volume of 100 c.c. During exposure small amounts of a colourless precipitate were deposited. At intervals this was filtered and washed and 10 c.c. portions of the clear solution were taken, the methyl alcohol was removed *in vacuo*, and the residue extracted with successive portions of low boiling petroleum ether, with gentle warming. The petroleum ether was evaporated in a current of carbon dioxide and the residue taken up with five portions of 2.5 c.c. methyl alcohol, with cooling and filtration of each fraction. To the combined methyl alcoholic extracts, 5 c.c. of *N* methyl alcoholic potash were added, and following saponification, determination of the pyrethrins made by the acid method.

The amounts of pyrethrins I and II present in 10 c.c. of the solution at different stages in the exposure were as follows:

Exposure	Pyrethrin I mg. per 10 c.c.	Pyrethrin II mg. per 10 c.c.
At start 0 hours	90.17	76.38
8 "	53.85	63.04
16 "	26.83	50.09
44 "	4.12	21.69
90 "	0.87	3.39

The logs of the pyrethrin figures are plotted against the times of exposure in Fig. 4.

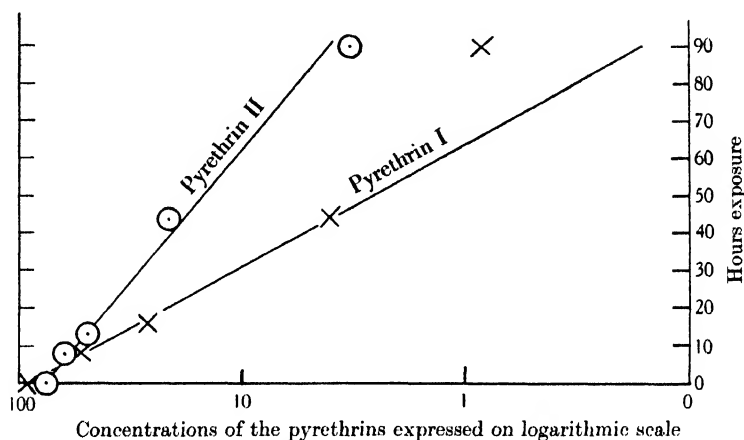


Fig. 4. Decline in pyrethrin content of aerated pyrethrum extract in methyl alcohol solution on exposure to sunlight.

It is seen that four out of the five points in the case of pyrethrin I, and five of the points for pyrethrin II fall very near to straight lines. The lag in the early stages of exposure was not appreciable. The last point for pyrethrin I shows a departure from the line, and we suspect that this discrepancy is probably due to non-pyrethrin material. If the remaining points on the diagram are corrected for this residual value, they still show a linear relationship. The loss of pyrethrin I under these conditions of exposure, as with the pyrethrinised dust, is more rapid than that of pyrethrin II, but we are unaware of the extent to which the reactions influenced each other. It is probable that valuable information on loss of toxicity may be obtained from further carefully controlled experiments on the exposure of the pyrethrins in solution.

Exposure of pyrethrum flowers to sunlight. In Table VIII are set out the data obtained for five samples of pyrethrum in different states of fineness of division, under different conditions of exposure.

Table VIII. *Exposure of pyrethrum flowers in various ways.*

Exposure	Finely ground, coarsely ground, and whole heads.									
	Harpenden		Dalmatian 1606 Open flowers		Dalmatian 1607 Open flowers		Dalmatian 1606 Half-closed		Dalmatian 1609 All closed	
	Pyre- thrin I	Pyre- thrin II	Pyre- thrin I	Pyre- thrin II	Pyre- thrin I	Pyre- thrin II	Pyre- thrin I	Pyre- thrin II	Pyre- thrin I	Pyre- thrin II
	FINELY GROUND.									
At start	0.73	0.78	0.19	0.21	0.26	0.31	0.30	0.26	0.43	0.38
90 days:										
Glasshouse open trays	0.16	0.46	0.05	0.20	0.05	0.23	0.09	0.17	0.14	0.23
Glasshouse covered trays	0.67	0.59	0.20	0.24	0.25	0.32	0.28	0.24	0.37	0.36
Glasshouse in tins	0.71	0.68	0.21	0.19	0.26	0.28	0.28	0.23	0.37	0.28
Cellar in tins	0.66	0.65	0.19	0.21	0.25	0.32	0.27	0.23	0.36	0.29
177 days:										
Glasshouse open trays	0.04	0.23	0.02	0.13	0.02	0.20	0.02	0.10	0.04	0.14
Glasshouse covered trays	0.65	0.70	0.19	0.20	0.25	0.30	0.25	0.23	0.34	0.32
Glasshouse in tins	0.65	0.66	0.18	0.25	0.26	0.31	0.28	0.25	0.35	0.36
Cellar in tins	0.67	0.69	0.19	0.23	0.26	0.31	0.26	0.25	0.36	0.35
365 days:										
Glasshouse open trays	0.02	0.11	0.01	0.06	0.01	0.06	0.01	0.05	0.03	0.07
Glasshouse covered trays	0.56	0.63	0.16	0.22	0.25	0.29	0.20	0.19	0.27	0.27
Glasshouse in tins	0.57	0.67	0.20	0.23	0.26	0.28	0.24	0.19	0.33	0.30
Cellar in tins	0.65	0.74	0.20	0.25	0.25	0.32	0.24	0.25	0.35	0.36
COARSELY GROUND.										
At start	0.66	0.63	0.16	0.19	0.24	0.25	0.28	0.24	—	—
90 days:										
Glasshouse open trays	0.20	0.42	0.06	0.16	0.09	0.21	0.08	0.12	—	—
Glasshouse covered trays	0.55	0.57	0.15	0.17	0.22	0.24	0.22	0.19	—	—
Glasshouse in tins	0.63	0.62	0.15	0.16	0.22	0.23	0.21	0.18	—	—
Cellar in tins	0.63	0.62	0.15	0.18	0.22	0.25	0.22	0.18	—	—
177 days:										
Glasshouse open trays	0.05	0.19	0.03	0.14	0.03	0.18	0.03	0.09	—	—
Glasshouse covered trays	0.60	0.60	0.15	0.17	0.22	0.27	0.20	0.18	—	—
Glasshouse in tins	0.61	0.60	0.15	0.20	0.22	0.26	0.22	—	—	—
Cellar in tins	0.59	0.62	0.15	0.19	0.22	0.28	0.22	0.21	—	—
365 days:										
Glasshouse open trays	0.02	0.09	0.02	0.07	0.02	0.08	0.01	0.03	—	—
Glasshouse covered trays	0.52	0.52	0.15	0.17	0.21	0.24	0.16	0.14	—	—
Glasshouse in tins	0.51	0.57	0.15	0.16	0.21	0.24	0.18	0.17	—	—
Cellar in tins	0.55	0.55	0.17	0.19	0.24	0.25	0.19	0.16	—	—
WHOLE HEADS.										
At start	0.73	0.78	0.19	0.21	0.26	0.31	0.30	0.26	0.43	0.38
90 days:										
Glasshouse open trays	0.59	0.79	0.19	0.22	0.23	0.26	0.18	0.20	0.38	0.37
Glasshouse covered trays	0.79	0.61	0.21	0.21	0.25	0.23	0.23	0.20	0.34	0.35
Incubator at 28° C.	0.72	0.66	—	—	—	—	—	—	—	—
365 days:										
Glasshouse open trays	0.44	0.60	0.11	0.15	0.18	0.23	0.18	0.18	0.31	0.27
Glasshouse covered trays	0.67	0.53	0.20	0.23	0.24	0.26	0.25	0.21	0.36	0.32
Glasshouse in tins	0.61	0.68	0.17	0.19	0.25	0.28	0.25	0.25	0.37	0.32
Cellar in tins	0.62	0.58	0.19	0.19	0.30	0.30	0.24	0.22	0.45	0.32
Incubator at 28° C.	0.64	0.65	—	—	—	—	—	—	—	—

Each of the samples of pyrethrum was divided into three parts. One part was ground to pass a sieve, the mean diameter of the holes of which was 0.584 mm., and the second ground to pass a sieve, the mean diameter of the holes of which was 1.72 mm. We have referred to these samples

respectively as finely and coarsely ground flowers. The third was left as whole heads. Each of these subsamples was again divided into four parts, one of which was exposed in a thin layer to direct sunlight in the glasshouse, a second was kept in trays in the glasshouse but protected by a cover from sunlight, the third in closed tins in the glasshouse, and the fourth in tins in a cool cellar. One sample of the Harpenden flowers (whole heads) was placed in a closed tin in an incubator kept at 28° C. The samples were stirred each week. The ground samples were tested at the end of 90, 177 and 365 days, and the whole heads at the end of 90 and 365 days. In the results given, no deduction has been made in the pyrethrin I estimation to allow for any residual non-pyrethrin value. The data show that with the fine and coarsely ground dusts loss of pyrethrin I is rapid in sunlight, there being little to distinguish the coarse from the finely ground sample. There is a slow loss in the case of the ground samples exposed in the covered trays and in the tins in the glasshouse, with the exception of the poor samples 1606 and 1607, where it is negligible. In the case of the whole heads, sampling is difficult, and errors due to this cause may be large, but the conclusion can be drawn that they lose their pyrethrin I at a very much slower rate than the ground flowers. Nevertheless, in the presence of sunlight the loss is definite both after 90 and 365 days. There would appear to have been a slight loss after 365 days in the whole flowers kept covered or in tins in the glasshouse. These results tend to confirm the data of Gnadinger and Corl⁽⁵⁾ who have pointed out that losses take place on storage in sealed containers.

Exposure of pyrethrinised dusts and pyrethrum powder in sealed flasks. An attempt to determine the extent to which oxygen is requisite to loss was made by exposing a pyrethrinised dust and ground pyrethrum powder in rubber-stoppered flasks in an atmosphere of nitrogen and air. 6 gm. of the dusts and powders were weighed in the dark into each of the flasks (volume 100 c.c.), which were then evacuated. Nitrogen, which had passed through several vessels containing alkaline pyrogallie acid, was slowly run in. The flasks were again evacuated and nitrogen again passed in, after which inlet and outlet tubes of each flask were sealed. In the case of exposure to an atmosphere of air, 6 gm. were weighed into each of a series of 100 c.c. flasks and these were closed with solid rubber stoppers. Each of the two sets of flasks was divided into three subsets, which were exposed in the following ways: (1) in a cool cellar in the dark, (2) to the light in a glasshouse, (3) in the dark at 28° C. in an incubator. The flasks were shaken at least every second day. Analyses were carried

out at the end of 8, 36, 72 and 196 days. No correction by deduction was applied to the estimation of pyrethrin I. The results are given in Table IX.

Table IX. *Exposure of pyrethrinised dust and pyrethrum powder in sealed flasks.*

Exposure	Nitrogen		Air	
	Pyrethrin I %	Pyrethrin II %	Pyrethrin I %	Pyrethrin II %
Pyrethrinised dust (pyrethrin I 0.46 %, pyrethrin II 0.45 %):				
Cellar (18° C.) 8 days	0.46	0.45	0.45	0.45
Glasshouse 8 days	0.40	0.41	0.22	0.38
Incubator (28° C.) 8 days	0.49	0.47	0.42	0.45
Cellar (18° C.) 36 days	0.44	0.40	0.43	0.38
Glasshouse 36 days	0.22	0.20	0.05	0.13
Incubator (28° C.) 36 days	0.42	0.41	0.43	0.39
Cellar (18° C.) 72 days	0.46	0.44	0.43	0.42
Glasshouse 72 days	0.21	0.22	0.05	0.16
Incubator (28° C.) 72 days	0.42	0.38	0.41	0.38
Cellar (18° C.) 196 days	0.42	0.44	0.42	0.46
Glasshouse 196 days	0.06*	0.10*	0.04	0.08
Incubator (28° C.) 196 days	0.37	0.35	0.24	0.25
Pyrethrum powder (pyrethrin I 0.45 %, pyrethrin II 0.49 %):				
Cellar (18° C.) 8 days	0.43	0.53	---	---
Glasshouse 8 days	0.39	0.49	0.34	0.48
Incubator (28° C.) 8 days	0.43	0.50	0.44	0.48
Cellar (18° C.) 36 days	0.42	0.43	0.40	0.43
Glasshouse 36 days	0.30	0.33	0.11	0.33
Incubator (28° C.) 36 days	0.39	0.44	0.39	0.41
Cellar (18° C.) 72 days	0.41	0.49	0.43	0.46
Glasshouse 72 days	0.30	0.37	0.07	0.33
Incubator (28° C.) 72 days	0.37	0.35	0.37	0.39
Cellar (18° C.) 196 days	0.42	0.47	0.41	0.49
Glasshouse 196 days	0.23	0.27	0.04	0.19
Incubator (28° C.) 196 days	0.37	0.41	0.37	0.40

* Air apparently had leaked in.

The figures for the estimation of pyrethrin I show that when pyrethrum dusts and powders are exposed in air to sunlight there is the usual rapid loss, but that the loss is somewhat slower in the case of powdered flowers than the artificially prepared dusts. The loss is definitely slower in an atmosphere of nitrogen than in air. We suspect that after 72 days there may have been a leakage of air into the flasks containing nitrogen and exposed in the glasshouse, as the values which had remained constant from 36 to 72 days declined more rapidly. We cannot be sure that a little oxygen was not occluded with the dusts nor do we know the part played by adsorbed moisture, but the experiment tends to show that free oxygen may not be requisite for slow losses. Comparisons of the results

obtained from exposure in tins in the incubator and in the cool cellar indicate that there is a greater loss at the higher temperature. Gnadinger and Corl⁽⁵⁾ have shown that activity of pyrethrum declines more slowly at low temperatures than at high temperatures. Our results confirm this finding and the determination of the temperature coefficient might be of interest.

Other experiments were carried out in glass bulbs of about 70 c.c. capacity, having inlet tubes running about half-way to the bottom, and outlet tubes sealed also into the top. 6 gm. of a pyrethrinised dust were weighed into each tube in the dark, and nitrogen was passed in after being freed from oxygen by running a fine stream of bubbles through a series of sintered glass gas-wash bottles containing strong alkaline sodium hydrosulphite solution. The tubes were then evacuated by a Hyvac pump and nitrogen again passed in. After repeating this process the tubes were sealed, and exposed for a period of 122 days. The tubes were shaken every two days. Table X gives the results:

Table X. *Exposure of pyrethrinised dusts in special tubes.*

Description	Pyrethrin I	Pyrethrin II
	%	%
Composition of dust at start: (1)	0.65	0.48
(2)	0.65	0.51
Dust in air exposed to sunlight after 122 days	0.17	0.22
Dust in nitrogen exposed to sunlight after 122 days	0.47	0.37
Dust in air in incubator in dark at 48° C. 122 days	0.40	0.36
Dust in nitrogen in incubator in dark at 48° C. 122 days	0.58	0.50
Dust in nitrogen in cool cellar in dark 122 days	0.64	0.56

Although it is impossible to be certain that no traces of oxygen were occluded in the tubes filled with nitrogen, the amount present must have been very small, and it is fairly evident from the above that losses of pyrethrin can take place in the presence of an inert gas, although at a much slower rate than in air, and that loss in nitrogen takes place in the incubator at 48° C. but again at a slower rate than in air. The dust exposed both to nitrogen and air in the incubator at the relatively high temperature of 48° C. suffers appreciable loss of pyrethrin I, although this is not so great in either case as in sunlight. The decline in pyrethrin I in nitrogen is greater at 48° than in the cool cellar (temperature approximately 18° C.) where the loss was found to be infinitesimal.

Chemical evaluation of exposed pyrethrinised dusts containing antioxidants. The antioxidants used in this trial were tannic acid and hydroquinone. The dusts were prepared by extracting Harpenden grown flowers (1932 crop) with low-boiling petroleum ether, the concentrated

extract was mixed with finely divided talc, and the petroleum ether removed *in vacuo* after thorough mixing. The dust was divided into three portions of 50 gm. each: to one was added 1.25 gm. of hydroquinone, another 1.25 gm. of tannic acid, while the third portion was kept as a control. The hydroquinone and the tannic acid were incorporated into the dusts in solution in the minimum amount of absolute alcohol, which was finally removed *in vacuo*. 5 gm. portions of the control and treated dusts were then exposed in shallow trays to a 1000-watt lamp, with stirring at regular intervals of half an hour. Different exposure periods up to 40 hours were used, and the pyrethrin I content of the dusts at the end of each exposure was determined. We have made no attempt to correct the figures for pyrethrin I. It is obvious from the data that these antioxidants have a stabilising effect upon activity and it is possible that this may be sufficiently marked to extend the use of pyrethrinised dusts. It is, however, questionable whether, owing to difficulties of thorough incorporation, the stabilising effect would be so noticeable with powdered flowers. The data are given in Table XI and expressed in Fig. 5.

Table XI. *Exposure of pyrethrinised dusts with and without antioxidants.*

Exposure hours	Pyrethrin I, % of dust		
	Control	Tannic acid	Hydroquinone
At start	0.59	0.45	0.57
5	0.46	0.40	0.52
10	0.28	0.36	0.49
15	0.08	0.33	0.44
20	0.04	0.26	0.34
30	0.03	0.15	0.24
40	0.04	0.10	0.17
Cellar in tins	0.61	0.47	0.58

Loss of colour of pyrethrinised dusts. The question has been raised from time to time whether the colour of ground pyrethrum flowers is in any way correlated with activity. We have noted, during our work on the exposure of dusts, that the colour began to fade in samples untreated by antioxidants many hours before a similar loss occurred in treated samples, and also that the fading of the colour was about complete in the same time as the pyrethrin I value required to fall to an almost negligible level. For example, after 40 hours' exposure there was a distinct loss of colour in the tannic acid treated dust and partial loss in the case of the hydroquinone treated dust, while the untreated dust was colourless.

The rapid loss in the colour of the untreated dust was followed by determining the changes in the absorption bands of a clear alcoholic

extract. The extract of the non-exposed dust showed a strong absorption band at 6750–6640 Å., very faint bands at 6100 and 5350 Å., while the violet end of the spectrum was extinguished from 5010 Å. The band in the orange region could not be seen after 12 hours, while, as exposure proceeded, the band in the violet region decreased gradually, disappearing entirely after 16 hours, when the dust appeared colourless. The points marked \times in Fig. 5 show the retraction of the broad band in the violet

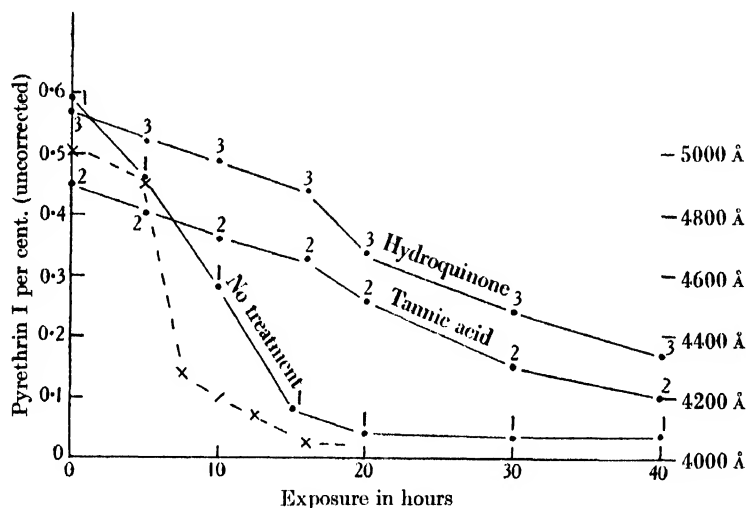


Fig. 5. Loss of pyrethrin I in pyrethrised dust with and without antioxidants on exposure to 1000-watt lamp.

- | | |
|----------------|---|
| ● ¹ | ● ¹ Untreated dust. |
| ● ² | ● ² Tannic acid treatment. |
| ● ³ | ● ³ Hydroquinone treatment. |
| \times | \times Retraction of absorption band. |

on exposure of the untreated dust. There was no attempt to measure by means of the spectrophotometer the decreasing intensity of the band, but the points indicate that during exposure the violet band had retracted to much smaller dimensions by the time that the pyrethrin I value had fallen almost to zero. It is doubtful, however, whether there is any rigid connection between colour and activity, but there is a possibility that certain plant pigments, owing to their structure, *e.g.* the anthoxanthins, might have a stabilising effect against oxidation. It is also probable that the factors making for loss of colour are closely related to those making for loss of activity in pyrethrum when the latter is exposed to light and air.

DISCUSSION.

An attempt has been made in the foregoing pages to submit an analytical criticism of some of the results obtained in a preliminary investigation (*loc. cit.*) of the causes of the loss of activity of pyrethrum, when exposed to light and air. One of the limiting factors in the use of pyrethrum as an insecticide has been the fugitive nature of its activity. In addition, we believe that the difficulties and discrepancies of investigation have been increased by the labile nature of the active principles.

The loss of activity of pyrethrised dusts on exposure may be followed with a good, but not absolute degree of accuracy by the determination of pyrethrin I. There is a small residual value left after exposure, with which the biologically determined insecticidal activity is not in strict correlation¹, and by deducting this residual value the concordance between the estimation of pyrethrin I and toxicity is increased. Although in the case of normal rich flowers and dusts the correction may be so small as hardly to affect the results and scarcely outside the degree of discrepancy of two different investigators, it may become of greater importance in the case of poor samples.

We have exposed both flowers and artificially prepared dusts in air to sunlight, and the dusts to the illumination of a 1000-watt lamp for varying periods, and find that, after an initial lag period, the rate of loss of activity is rapid and then decreases. It was found that in a methyl alcoholic extract of the flowers, exposed to a rapid stream of air in the presence of strong sunlight, the lag period was scarcely evident. Considering the loss in solution of pyrethrins I and II separately, the data over a large part of the exposure period corresponded in each case fairly closely with an unimolecular reaction, the rate of loss of pyrethrin II being slower than that of pyrethrin I. This conclusion, however, will require confirmation. When flowers are exposed to sunlight and air we were able to confirm the fact that powdered heads lose their activity more rapidly than whole heads. It was found that, although light accelerates loss by some process of activation, losses could take place in the dark and that there was apparently a temperature coefficient. Gnadinger and Corl⁽⁵⁾ have found that, at a low temperature, loss of activity is practically negligible. In a series of experiments in which powders and artificially prepared dusts were sealed in nitrogen it was found by us that loss of pyrethrin I took place, although at a materially slower rate than in air. Furthermore, in the presence of nitrogen, the loss of pyrethrins seems to take place more rapidly at higher than at lower temperatures and also to

¹ The nature and extent of this apparent discrepancy is now being examined.

be accelerated by light. It is recognised that to free our dusts and powders from the last traces of oxygen may not be easy, but it appears that pyrethrum may degenerate by processes other than oxidation by *free* oxygen. The presence of adsorbed moisture in this connection is probably of importance.

Both by biological trials and analytical tests we have confirmed the partial stabilising effect of the addition of antioxidants, but the suggestions that antioxidants might lead to an enhanced toxicity of the pyrethrins in sprayed pyrethrum extracts was not confirmed. The fading of the colour of pyrethrinsed dusts in light and air and its partial stabilisation by the incorporation of antioxidants have been noticed. The gradual contraction of the broad band in the violet end of the spectrum was observed to run roughly parallel with the loss of pyrethrin I. Although no direct connection can be traced between the degree of colour and activity, and between loss of colour and loss of activity, it would appear as if the same causes were leading to these parallel effects. Whether any of the naturally occurring colour pigments, either through an antioxidation effect or through preferential absorption of light, lead to a stabilising action is a matter of some interest. So far, with the few synthetic dyes experimented with, we have not been able to secure results of a definite nature.

SUMMARY.

1. The loss of activity of pyrethrum flowers and preparations has been studied.
2. The degree of concordance between the content of pyrethrin I, as determined by the acid method, and the insecticidal value of pyrethrinsed dusts, before and after exposure to air and artificial illumination, has been statistically examined. The pyrethrin I values, corrected for a small residual amount of extraneous matter, indicate fairly closely the degree of activity of the samples. The loss of pyrethrins on exposure has been traced out quantitatively.
3. A comparison was made between two samples of pyrethrum flowers, one rich and the other poor in pyrethrins, in order to determine the degree of concordance between the pyrethrin I content and their toxicity. The pyrethrin I value as determined by the acid method, subject to a small correction, gave a good indication of the relative activities of the samples.
4. Pyrethrum flowers, both finely and coarsely ground and as whole heads, have been exposed under various conditions for different periods up to one year. There was a relatively rapid loss of pyrethrin I in cases

where the ground flowers were exposed to sunlight and air, but when stored in covered trays or tins the loss of pyrethrin I was much slower. The pyrethrins undergo change at a slower rate in flowers stored as whole heads than in the ground state.

5. Pyrethrised dusts and ground flower heads lose their pyrethrin content when exposed to sunlight in an atmosphere of air or of nitrogen. The loss in nitrogen is less rapid than in air and appears to be due to a reaction other than oxidation by free oxygen. The effect of temperature upon the rate of loss of the pyrethrins is shown.

6. The rate of loss of the pyrethrins in a methyl alcoholic extract of flowers, when exposed to sunlight and air, was studied.

7. The stabilising effect of tannic acid and hydroquinone when added to a talc pyrethrum dust was confirmed. It was shown that such mixtures lose their pyrethrins at a slower rate when exposed in thin layers to air and artificial illumination. Biological trials showed that the addition of these antioxidants did not augment the initial insecticidal activity of the pyrethrins.

We have found during the course of this work that Dr C. I. Bliss's system of probits has afforded us a powerful and simple device for the statistical analysis of our results. We wish to express to him our thanks for advance copies of his tables and for his advice. We wish also to acknowledge with our thanks the help and assistance afforded to us by Mr P. C. Bowes, Mr A. Ogglesby and Miss I. P. Randall. We are also indebted to Messrs Stafford Allen and Co., Ltd., for kindly supplying us with Dalmatian samples of pyrethrum.

REFERENCES.

- (1) BLISS, C. I. *Science* (1934), **79**, 38, 409.
- (2) FISHER, R. A. *Statistical Methods for Research Workers* (1930), 3rd ed. London: Oliver and Boyd.
- (3) GADDUM, J. H. *Reports on Biological Standards* (1933), Mod. Res. Council Special Rep. Series, No. III, No. 183.
- (4) GNADINGER, C. B. *Pyrethrum Flowers* (1933). Minneapolis: McGill Lithograph Co.
- (5) GNADINGER, C. B. and CORL, C. S. *Ind. Eng. Chem.* (1932), **24**, 901.
- (6) HEMMINGSEN, A. M. *Quart. J. Pharm.* (1933), **6**, 39.
- (7) MCCALLAN, S. E. A. and WILCOXON, F. *Contrib. Boyce Thompson Inst.* (1933), **5**, 173.
- (8) TATTERSFIELD, F. *J. Agric. Sci.* (1932), **22**, 396. (Rothamsted Memoirs, 17.)
- (9) TATTERSFIELD, F. and MORRIS, H. M. *Bull. Ent. Res.* (1924), **14**, Pt. 3, p. 223. (Rothamsted Memoirs, 12.)
- (10) TATTERSFIELD, F., HOBSON, R. P. and GIMINGHAM, C. T. *J. Agric. Sci.* (1929), **19**, 266. (Rothamsted Memoirs, 14.)

(Received June 27th, 1934.)

AN APPARATUS FOR TESTING CONTACT INSECTICIDES.

By F. TATTERSFIELD, D.Sc., F.I.C.

(*Department of Insecticides and Fungicides, Rothamsted
Experimental Station, Harpenden, Herts.*)

(With Plate XXXVI and 5 Text-figures.)

CONTENTS.

	PAGE
Introduction	691
Description of atomiser No. 1	692
Delivery of spray from type atomiser No. 1	693
Distribution of spray by atomiser No. 1	694
Atomiser No. 2	697
Distribution of spray by atomiser No. 2	697
The effect of reducing the opening of the needle valve on the distribution of the spray	698
The effect of increasing the depth from the atomiser on the distribution of the spray	700
Summary	702
References	703
Explanation of Plate XXXVI	703

INTRODUCTION.

IN 1924 Tattersfield and Morris(2) published an account of an apparatus suitable for spraying insects with contact insecticides under laboratory conditions. This apparatus has been in use since that date and has proved satisfactory for its purpose. The atomiser used in this model was so constructed that a current of air was blown over the end of a capillary tube dipping into a reservoir containing the solution or suspension of the insecticide to be tested; the fluid was drawn from the reservoir and thrown into a fine spray over the insects. The data given in the previous paper showed that the sprayings could be replicated with considerable exactitude, and insecticide tests carried out gave sigmoid curves, relating concentration of poison to percentage number of insects rendered moribund or dead, now regarded as typical for the relation of dosage and effect. The distribution of the spray over the area upon which the insects were placed was comparatively uniform at the depth of the dish from the nozzle used. When, however, replicates of the atomiser had to be con-

structed it was found no easy matter to make the necessary adjustments between the orifices through which the air was blown and that of the tube connected with the reservoir. Although this type of atomiser, in which each minute drop, as it emerges, is scattered by an air blast at right angles to the path of the drop, results in considerable evenness in the distribution of the spray, it unfortunately affords no opportunity for regulating the fineness of division of the spray or the time required for delivery. Messrs Pellant and Son of Harpenden have recently designed and made for us two types of atomiser, suitable for operation by compressed air, in which the air current dispersing the drop is in line with the path of the latter, as it emerges from the orifice of the tube connected with the reservoir. By the use of a needle valve, the size of

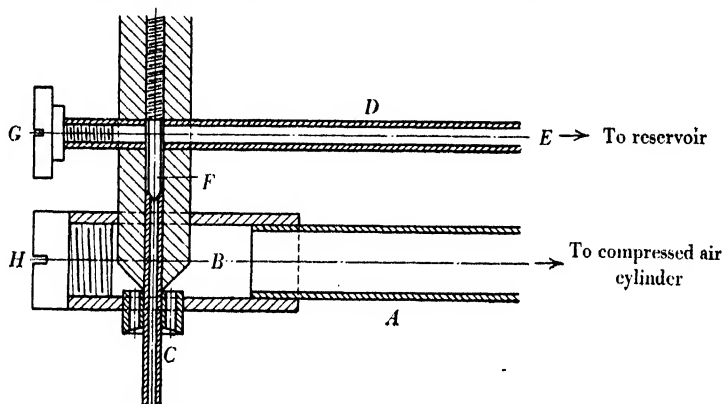


Fig. 1. Vertical section through atomiser No. 1 (twice actual size).

the actual spray droplets and the time taken in spraying any given amount can be regulated. This paper gives an account of these new types of atomiser and the distribution of spray given by them.

DESCRIPTION OF ATOMISER NO. 1.

A plan of atomiser No. 1 is given in vertical section in Text-fig. 1. It consists essentially of a brass tube of narrow bore *D* extended beyond *E* and bent at right angles to dip into a small glass reservoir; at the other end the tube is brazed into a stout brass tube of narrow bore as shown, into the lower end of which a capillary brass tube is brazed; this fine tube can be partially or wholly closed by the needle *F*, worked by a screw with milled head. The extension *G*, fitted with a screw, affords a means of clearing *D* if it becomes clogged, either by blowing air through or by means of a fine wire. The tube *A* which leads from the air cylinder,

fits tightly into the chamber *B*, the compressed air passing through three orifices, two of which are shown at *C*. These openings are cut so that the outflow of air rushes as closely as possible along the jet carrying the liquid. The opening closed by a screw at *H* permits of clearing tube *B* by blowing a little air through from the compressed air cylinder.

DELIVERY OF SPRAY FROM TYPE ATOMISER No. 1.

The atomiser is fixed into a clamp, which allows such movements as are requisite for centring the spray. The clamp is attached by pins to the brass plate let into the wooden lid (see Plate XXXVI), which can be made to fit accurately a large glass cylinder 20 cm. in diameter by means of movable blocks. The jar, which is supported on a levelling platform, is fitted inside with a second brass platform with levelling screws and ivory-tipped screws for holding it firmly in a central position. (By means of marks on the jar the platform can always be returned to the same position after removal.) The internal platform can be made to take dishes of different sizes by means of adjustable uprights. The platform and atomiser are so adjusted with respect to each other that the centre of the former falls directly under the tip of the atomiser. A gallows arrangement with adjustable plummet with pointed end is attached to the atomiser; under the pointed end of the plummet is fixed a piece of squared paper, which serves to indicate any movement of the atomiser. The cone of the spray is centred by lowering into the jar a circular glass plate with a central hole through it to facilitate removal; on this glass plate a circular disc, of the size of the dish to be used for spraying, is placed with its centre over the centre of the platform. 1 c.c. of a solution of saponin in water (0.5 or 1.0 per cent.) is pipetted into the reservoir attached to arm *D* of the atomiser. The tube *A* is connected as shown in the plate through a pressure gauge and air filter to a compressed air cylinder, adjusted to deliver air at known pressure. The lid having been placed in the marked position on the jar, the air is turned on and the liquid in the reservoir is thrown in a fine spray over the plate. The operation is repeated with suitable adjustments of the atomiser until the distribution of the spray is symmetrically disposed about the smaller disc. When adjusted, the clamps are screwed up tight, to prevent further movement. As the brass plate contains two pegs fitting accurately through the plate of the clamp bearing the nozzle, the latter can be taken out and replaced if required without greatly modifying the distribution of the spray. This operation, however, should be carried out as seldom as possible and the distribution of the spray re-checked afterwards.

The above procedure enables one to obtain as even a distribution as is usually necessary in spraying technique; for more accurate work, however, the distribution can be gauged by lowering into the jar over the platform a large glass disc, to the lower side of which is attached a sheet of paper ruled in concentric circles. A small dish of known diameter can then be placed in positions at varying distances from the centre and the amount of spray falling upon it determined by weighing.

The data for the weight of spray falling on a dish 19.63 sq. cm. in area when the cone of spray was symmetrically disposed and the needle valve open to different amounts are given in Table I.

Table I.

Amount of spray falling upon dish 19.63 sq. cm. internal area. 1 c.c. water sprayed.

Distance of jet from dish 31.0 cm.		Spray atomiser No. 1.	
Valve open	No. of sprayings	Mean weight of spray (gm.)	Standard error
Fully open = 2 mm.	8	0.4445	± 0.0006
Half-open = 1.0 mm.	10	0.4461	± 0.0012
One-quarter open = 0.5 mm.	5	0.3871	± 0.0029

These data show that the cone is comparatively narrow and that little difference is made to the dispersion of the spray by the change of the needle valve from half-open to fully open, although reduction to one-quarter open gives the distribution a slightly wider angle. In actual trial it was found that the amount of liquid delivered is rather too great, and it was evident that means would be needed for scattering the spray more efficiently.

DISTRIBUTION OF SPRAY BY ATOMISER NO. 1.

For determining the evenness of distribution of spray the method described was employed. A circular sheet of paper was ruled with concentric circles 2.45 cm. apart, and in addition small circles (the size of the dish to be used for weighing the spray) were described, radiating from the centre at angles of 60°. The paper was attached by suitable adhesive to the underside of the circular glass plate. The spray was centred in the usual way, and the weights of spray falling upon a small glass dish 2.7 cm. external diameter with walls 0.65 mm. deep were determined by weighing. Three solutions were sprayed: (1) distilled water, (2) a half per cent. solution of saponin in water, (3) a half per cent. solution of saponin containing 5 c.c. per 100 c.c. of absolute alcohol (a solution frequently used by us in spraying technique). The needle valve was in

the fully open position, that is it was raised 2 mm. The data are given with standard errors in Table II.

Table II.

*Weights of spray falling upon a dish 5.725 cm.² in area
at a depth of 31 cm.*

Spray used	Mean weight of spray (gm.) at a distance (cm.) of centre of dish from centre of plate of					
	0 (centre)	0.67	1.35	2.7	5.4	8.1
Water	0.1293 ±0.0026	—	0.0946 ±0.0037	0.0368 ±0.0022	0.0086 ±0.0005	0.0036 ±0.0006
Saponin solution 0.5 %	0.1242 ±0.0020	0.1159 ±0.0016	0.0968 ±0.0028	0.0533 ±0.0021	0.0097 ±0.0007	—
Saponin solution 0.5 % + 5 % by vol. alcohol	0.1208 ±0.0016	0.1133 ±0.0034	0.0921 ±0.0048	0.0457 ±0.0033	0.0092 ±0.0003	—

Although, as the values for the standard errors show, there was some variation in the weights of spray falling upon the dish in different positions in the same concentric circle, there was no certain reason for considering that this was due to the spray not being symmetrically disposed with respect to the dish, rather than to experimental error. The figures are plotted in Text-fig. 2.

The data in Table II and the curves demonstrate that with this atomiser, although there is only a slight change in the distribution of the spray when saponin solution and saponin solution containing alcohol are used instead of water, there is a tendency to a wider scatter of droplets when the surface tension is reduced. The radius of the dish usually used for spraying purposes is about 2.5 cm. The data and the curves show that this atomiser is *not* eminently suitable for the quantitative spraying of insects scattered at random over an area of about 20 sq. cm., owing to the rapid diminution of the amount of spray deposit with an increase in distance from the centre. The atomiser has, however, been used in certain spray trials and, provided 1 c.c. was sprayed and a large enough number of insects were employed, quantitative comparisons could be obtained. The curves obtained by plotting dosage against kill were S-shaped and reduced to a straight line when Bliss's probits method (1) was employed. The biological results were not very satisfactory when 0.5 c.c. of spray fluid was substituted for 1 c.c. It is considered that the spray which amounted to 0.4 c.c. over the area of the dish (nearly twice as much as the atomiser in the original machine) is too great in amount. The large quantity of spray falling on the dish led to a greater percentage kill for the same concentration of poison than was obtained

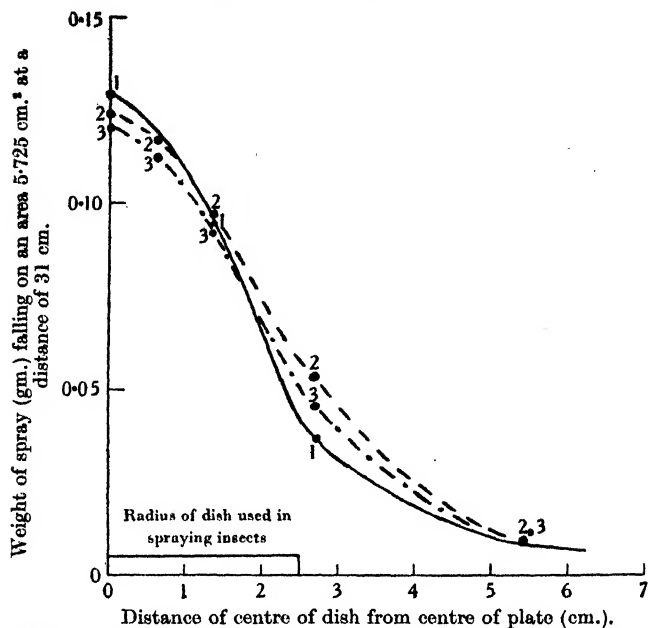


Fig. 2. Distribution of spray. Atomiser No. 1. Valve fully open.

●¹ — ●¹ Water. ●² — — ●² 0.5 % saponin solution.
 ●³ — · — · ●³ 0.5 % saponin + 5 % by vol. of alcohol.

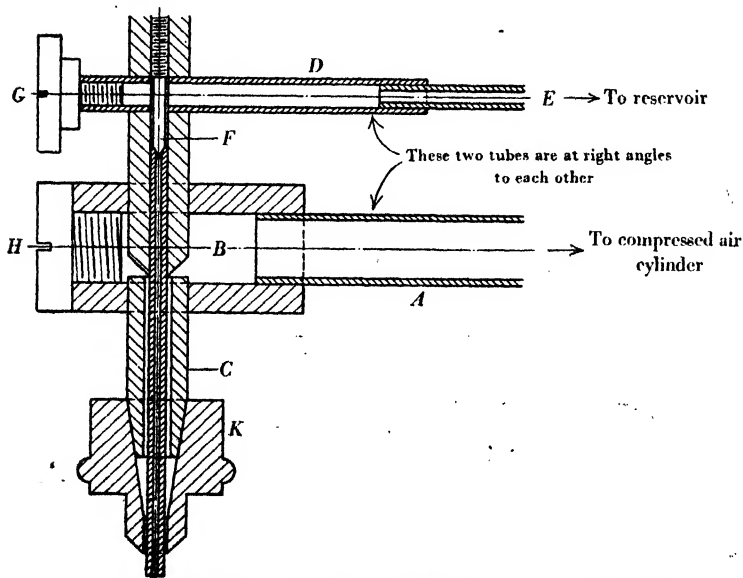


Fig. 3. Vertical section through atomiser No. 2 (twice actual size).

when the original atomiser was used. In view of these facts attempts were made to improve the evenness of the distribution over the surface of the dish.

ATOMISER No. 2.

Mr F. W. Pellant designed another atomiser, which could not only be replicated more easily than the one described above, but gave a distribution of spray comparable with the original form. Its design is shown in Text-fig. 3.

The design of atomiser No. 2 differs from that of No. 1 in one important particular. The brass tube *C* tapers externally to take and hold by friction a spray cap *K*¹. Internally this cap has a conically cut bore, and the air emerges from it much nearer to the orifice of the tube conducting the spray fluid; this leads to a greater scatter of the spray droplets. The other details of the atomiser are much the same as in the first model, *e.g.* the screw caps *G* and *H* can be detached, if it is found necessary, to clear the tubes *A* and *D*, either by blowing air down the tubes or by the insertion of a thin wire.

DISTRIBUTION OF SPRAY BY ATOMISER No. 2.

The amount of spray delivered on to the surface of a dish 5.2 cm. external diameter (=21.24 sq. cm. in area) placed at two different depths from the orifice of the atomiser, when 1 c.c. is sprayed, and when the needle valve is one turn, one half-turn or one-quarter turn open is shown in Table III.

Table III.

Weight of spray delivered on to an area of 21.24 cm.²
(5.2 cm. diameter) by atomiser No. 2, 1 c.c. sprayed.*

Spray fluid: 0.5 per cent. saponin solution containing 5 % by vol. of alcohol.

Depth of dish from atomiser cm.	Needle valve open	Weight of spray gm.	Ratio of weights of spray	Ratio (31.5) ² : (35.3) ² = 0.7962
31.5	One full turn	0.2445 (1)	(1):(2) = 1.1283	—
	One-half turn	0.2167 (2)	(2):(3) = 1.0917	—
	One-quarter turn	0.1985 (3)	—	Ratio (4):(1) = 0.7935
35.3	One full turn	0.1940 (4)	(4):(5) = 1.1312	Ratio (5):(2) = 0.7914
	One-half turn	0.1715 (5)	(5):(6) = 1.0910	Ratio (6):(3) = 0.7919
	One-quarter turn	0.1572 (6)	—	—

* This area differs slightly from the area of dish in Table I as the weight falling upon the external area of dish was taken.

The data in Table III show that atomiser No. 2 delivers less into the dish at about the same depth than atomiser No. 1, the amount being very

¹ It is of considerable importance to have the cap *K* concentrically disposed round the orifice.

nearly the same with the valve fully open at a depth of 31.5 cm. as the original type of atomiser formerly gave at 35-6 cm., but rather less when the depth is increased to the latter figure (2). The amount delivered is nearly inversely proportional to the square of the depths of the dish from the orifice of the atomiser (column 5). There is, however, a slight discrepancy, which indicates that the evenness of scatter is slightly greater at the greater depth and that either the cone of spray is slightly curved outwards, or, more probably, that the concentration of the spray in the centre is more thoroughly broken up as the depth increases. Since two different glass jars were used in these tests, it is evident that the atomiser can be centred with considerable accuracy, if care be taken in the operation. Data obtained by a further investigation on the effect of depth upon the evenness of the spray are given below. The ratios of the weights of spray when the needle valve was open to different degrees are given in column 4, and it is shown that the ratios are the same for the two depths when comparisons are made between the valve fully open and half-open and quarter-open, that is, for both depths the same change in the degree of openness of the needle valve introduced proportionally an equivalent change in the degree of scatter.

THE EFFECT OF REDUCING THE OPENING OF THE NEEDLE
VALVE ON THE DISTRIBUTION OF THE SPRAY.

The data in Table III indicate that the reduction from one full turn to one-half turn of the needle valve increases the degree of scatter, a further reduction to one-quarter still further increases it, but to a somewhat less extent. The effect upon the distribution of the spray was further studied with the circular plate ruled in concentric circles, by weighing the deposit on a small dish placed consecutively at greater distances from the centre in one direction. The data with the standard errors of the means of five determinations in each position, except the outermost one, are given in Table IV and plotted in Text-fig. 4.

The figures in Table IV confirm the deduction drawn from Table III that this atomiser delivers less in the centre of the cone of spray than atomiser No. 1, and that there is a much greater degree of scatter and thus that the distribution falls off less rapidly as the distance from the centre is increased. The degree of scatter is increased, as the needle valve is more nearly closed, and also as the pressure is increased from 15 to 24 lb. per sq. in. The proportional effects are shown by the ratios; thus, when the figures are taken for the weights of the deposits given with the needle valve open divided by those when half closed, although the deposit

at the centre is 1.23 times greater with the open valve than with the half-open, it has become nearly unity at a distance from it of 2.4 cm. and much less (0.716) at 4.8 cm. A similar kind of relationship exists between the values for the half-open and the quarter-open position, but the figure for the ratio after being nearly unity at a distance from the centre of 2.7 cm. rises again at 5.4 cm. Although the standard errors show that the result is scarcely significant, it indicates that the valve in an almost closed position apparently gives rise to some loss as a particulate cloud which either escapes from the jar or deposits only very slowly. The weights of the deposits are plotted against the distance of the centre of the dish from the centre of the plate in Text-fig. 4.

Table IV.

The effect on the distribution of spray of different valve openings. Atomiser No. 2.

Spray fluid: solution of 0.5 per cent. saponin in water containing 5 per cent. by volume of alcohol. Area of dish 5.725 cm.². Distance from orifice 31 cm. Pressure 15 lb./sq. in. and 24 lb./sq. in.

Weight of spray deposit (gm.) at a distance (cm.) of centre of dish from centre of plate

Needle valve open	Centre	1.35	2.7	5.4	8.1
Pressure 15 lb./in. ²					
One full turn (1)	0.0749 ± 0.001	0.0655 ± 0.0007	0.0435 ± 0.0013	0.0078 ± 0.0003	0.0014
One-half turn (2)	0.0610 ± 0.001	0.0559 ± 0.0013	0.0404 ± 0.0012	0.0109 ± 0.0011	0.0029
One-quarter turn (3)	0.0535 ± 0.0004	0.0519 ± 0.0004	0.0414 ± 0.0004	0.0089 ± 0.0004	0.0024
Pressure 24 lb./in. ²					
Half turn (4)	0.0535 ± 0.0005	0.0469 ± 0.0012	0.0343 ± 0.0008	0.0117 ± 0.0004	0.0069
Ratio (1) : (2)	1.228	1.172	1.077	0.716	0.483
" (2) : (3)	1.134	1.077	0.976	1.225	1.208
" (2) : (4)	1.140	1.192	1.178	0.932	0.420

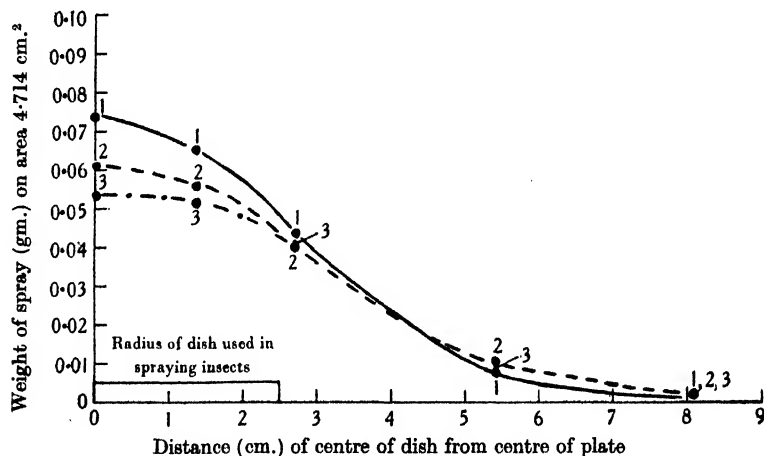


Fig. 4. The effect on the distribution of spray given by atomiser No. 2 by the degree of openness of the needle valve. ●¹—●¹ Valve open one full turn. ●²—●² Valve open one-half turn. ●³—●³ Valve open one-quarter turn.

THE EFFECT OF INCREASING THE DEPTH FROM THE ATOMISER
ON THE DISTRIBUTION OF THE SPRAY.

An examination of Text-fig. 5 shows that the weight of the deposit of spray begins to fall off rather rapidly at a distance of about 2 cm. from the centre of the circle of spray. It was decided to study the effect of increasing the depth upon the distribution, particularly within the area covered by the dish used in actual spray trials upon insects. For this purpose the same plate was employed as in previous experiments, but a flat-bottomed glass dish of external diameter 1.1 cm. was used; the walls of the dish were relatively high (0.5 cm.) with respect to the diameter, and although this may have introduced some error it is probable that it is nearly constant for the several positions, and the dish has the advantage over a disc of partially inhibiting splashing out and in ease of handling. The dish was weighed before each spray trial in a weighing bottle, and after spraying, carefully lifted with forceps, dried on the bottom, reintroduced into the weighing bottle and weighed again. Two different glass jars were used for spraying, which was carried out at depths of 30.5 and 34.0 cm. with the valve open to different degrees. The distribution of the spray was determined in directions changing consecutively by 60°. When the complete data were obtained for the depth 30.5 cm. with the open valve it was found that centring was not perfect; the true centre was then found and the distances from it corrected. For subsequent spraying the glass plate inside the jar was moved so as to have its centre in the centre of the circle of spray deposit. The experimental error is relatively large owing to the small weight of deposit, and the data are only given as showing the order of the distribution at the two depths. There is no evidence to be drawn from the full data that, when correct centring had been obtained, the weight of deposit at different points equidistant from the centre differed by more than the experimental error. We have tabulated and plotted the data for directions to right and left of the centre when a view is taken of the apparatus at right angles to the one shown in C, Plate XXXVI. The results are shown in Table V and Text-fig. 5.

Table V and Text-fig. 5 show that increasing the distance of the dish from the orifice improves the even nature of the distribution of the spray over an area equal to that used in practice for spraying insects, and that it is comparable with that obtained with the original atomiser. One would expect that it would be further improved by lowering the dish to the depth at which it is intended to use it, namely 35.3 cm. Increasing

Table V.

Distribution of spray over area of dish used in spraying insects at different positions of needle valve and at different depths.

Spray fluid: 0.5 per cent. solution of saponin + 5 % by vol. of alcohol.

Weight of spray (gm.) falling on dish 0.95 cm.² in area at a distance (cm.) of centre of dish from centre of plate

	Left of centre			Centre	Right of centre		
	cm.	cm.	cm.		cm.	cm.	cm.
Depth 30.5 cm.*:	1.95	0.85	0.3	—	0.8	1.9	2.45
Valve one full turn	0.0118	0.0158	0.0173	—	0.0167	0.0116	0.0096
Depth 30.5 cm.:	—	2.2	1.1	—	1.1	2.2	—
Valve one-half turn	—	0.0110	0.0118	0.0147	0.0129	0.0110	—
Valve one-quarter turn	—	0.0097	0.0122	0.0135	0.0126	0.0114	—
Depth 34 cm.†:	—	—	—	—	—	—	—
Valve one full turn	—	0.0098	0.0119	0.0128	0.0109	0.0113	—
Valve one-half turn	—	0.0075	0.0088	0.0109	0.0100	0.0093	—
Depth 34 cm.:	—	—	—	—	—	—	—
Original atomiser	—	0.0096	0.0101	0.01145	0.0099	0.0077	—

* These data are corrected to the true centre.

† Six replications taken round the centre gave mean values: centre 0.0133 ± 0.0003 gm., at 1.1 cm. 0.0118 ± 0.0004 gm., and at 2.2 cm. 0.0108 ± 0.00035 gm. (valve open one full turn).

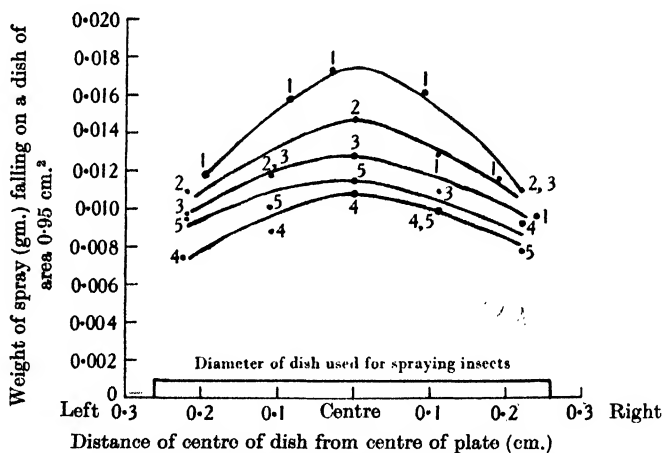


Fig. 5. Distribution of spray over an area 21.2 cm.² at two different depths.

- 1 — 1 Valve one full turn. Depth 30.5 cm.
- 2 — 2 Valve one-half turn. Depth 30.5 cm.
- 3 — 3 Valve one full turn. Depth 34 cm.
- 4 — 4 Valve one-half turn. Depth 34 cm.
- 5 — 5 Original atomiser. Depth 34 cm.

the distance from the atomiser has a very similar effect to a partial closing of the needle valve. The ratios of the weights falling upon the dish at the two different levels do not agree so well with that of the inverse square of the distance as when the larger area was used (Table II), but this is to be expected, as over the smaller area the experimental error and the relative height of the walls of the dish are greater. The ratio of the squares of the depths from the orifice is $(30.5)^2:(34)^2=0.80$. With the valve open one turn, the ratio of the weights at the centre is approximately 0.74, and with the valve open one-half turn it is 0.73; with the valve in the latter position the ratios at 1.1 and 2.2 cm. from the centre are 0.76. As the weight falling on an area of 0.95 sq. cm. at the periphery of an area of a diameter 5.2 cm. (equal to that upon which the insects are sprayed) only differs from that at the centre by about 2.5–3 mg. at a depth of 34 cm. from the orifice, and will be less at 35.3 cm., it would appear that this atomiser could be safely relied upon for quantitative spraying, if distances of this order or more are used. An objection to using much greater depths arises from the possibility of eddy currents making quantitative delivery of the spray a matter of difficulty.

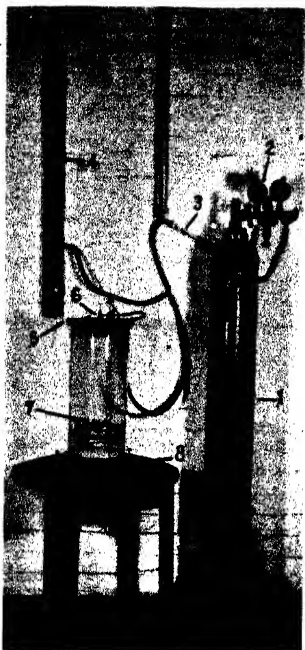
The atomiser can be made without the needle valve, and this may obviate any creeping of the spray fluid into the screw of the latter during actual spraying. A spraying chamber of square section, with a fixed lid and sliding door on one side for putting in the dish containing the insects, has also been constructed; the spray distribution is not affected by this form of apparatus, and although not so easy to wash out after use, it may have an advantage over a circular jar with movable lid in that there is less risk of inadvertently altering the position of the atomiser.

SUMMARY.

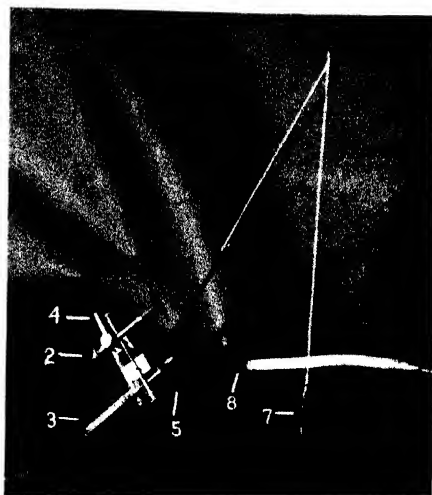
1. A description is given of the design of two atomisers for use in quantitative laboratory spraying for testing contact insecticides.

2. Tables and graphs are given showing the weights of spray delivered by each form upon known areas placed in different positions inside the spray jars, and an examination is made of the change in the distribution of the spray with the progressive closure of the orifice and increase in the distance from the orifice of the surface sprayed.

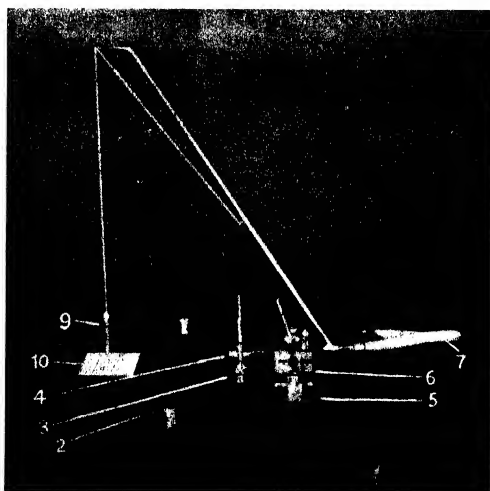
I am greatly indebted to Mr P. C. Bowes, formerly of this laboratory, for determining the distributions with atomiser No. 1, and especially to Mr F. W. Pellant, who designed and made the two atomisers and has supplied the drawings of them.



A. General view of apparatus.



B. Atomiser No. 2, clamp and plate detached from lid.



C. Near view of lid with atomiser No. 2 in position.

REFERENCES.

- (1) BLISS, C. I. (1934). The method of probits. *Science*, LXXIX, 38, 409.
(2) TATTERSFIELD, F. and MORRIS, H. M. (1924). *Bull. Ent. Res.* XIV, 223.

EXPLANATION OF PLATE XXXVI.

- A. Complete apparatus, \times about 1/20.
1. Cylinder of compressed air.
 2. Pressure regulating valve.
 3. Air filter.
 4. Pressure gauge.
 5. Removable lid of jar.
 6. Atomiser.
 7. Internal platform (with dish) supported by glass plate on glass tripod.
 8. External levelling platform.
- B. End view of atomiser No. 2, with brass plate and clamp removed from lid, \times about 1/4.
1. Milled head for adjusting needle valve.
 2. Orifice of atomiser No. 2 (showing cap).
 3. Glass reservoir with tube leading to atomiser.
 4. Brass plate removed from lid (atomiser is attached by clamp).
 5. Clamp.
 6. Gallows.
 7. Plummot.
 8. Tube leading to compressed air cylinder.
- C. Near view of lid with atomiser in position, \times about 1/4.6.
1. Milled head for adjusting needle valve.
 2. Screws for locking adjustable blocks to give lid a firm fit on jar.
 3. Top of glass reservoir.
 4. Atomiser.
 5. Brass basal plate on which removable plate and clamp are held.
 6. Removable brass plate with clamp.
 7. India-rubber pressure tubing leading to compressed air cylinder.
 8. Gallows.
 9. Plummot.
 10. Squared paper.

(Received May 25th, 1934.)

[FROM THE ANNALS OF APPLIED BIOLOGY, Vol. XX, No. 3,
pp. 404-420, August, 1933.]

[All rights reserved]

AN EXPERIMENT ON THE INCIDENCE AND SPREAD OF ANGULAR LEAF-SPOT DISEASE OF COTTON IN UGANDA

BY C. G. HANSFORD, M.A.

(Department of Agriculture, Kampala, Uganda),

H. R. HOSKING, B.Sc., A.R.C.S.

(Department of Agriculture, Serere, Uganda),

R. H. STOUGHTON, D.Sc.

(Department of Mycology, Rothamsted Experimental Station, Harpenden),

AND

F. YATES, B.A.

(Department of Statistics, Rothamsted Experimental Station).

(With 8 Text-figures.)

CONTENTS.

	PAGE
I. Design of the experiment	404
II. Course of the disease	406
(1) Primary infection	406
(2) Secondary infection	408
(3) Black-arm or stem infection	412
III. Influence of meteorological conditions	413
IV. Statistical analysis of results	415
V. Discussion	419
VI. Summary	420
References	420

I. DESIGN OF THE EXPERIMENT.

THE experiment to be described was designed to test the possibility of investigating the incidence and spread of the angular leaf-spot disease of cotton under field conditions, and of obtaining results which should be susceptible of statistical treatment. A great part of the knowledge of the dissemination of the disease and the influence of climatic conditions on this spread is purely empirical or deduced from laboratory studies, and this experiment, which is the first of a series, was undertaken to explore

the difficulties of putting this knowledge on a more exact foundation. It is intended to continue these experiments, modifying them from year to year as experience shows to be necessary, and thus to build up a collection of data from which definite conclusions may be drawn. The form and lay-out of the experiment was designed at a conference at Rothamsted in 1931, and duplicate experiments were carried out at Kampala and at Serere in Uganda during the cotton season 1931-2. Certain difficulties arose at Serere in the taking of the records, chiefly due to pressure of work, and in addition the spread of the disease at this station was so rapid as to obscure differences between plots very early in the season. For these reasons it has been thought best to confine attention in this paper mainly to the Kampala experiment, giving a summary only of the salient points from the Serere results.

The lay-out of the experiment at Kampala was in the form of a 4×4 Latin square, chosen by random selection (see Fig. 1). Each plot consisted of eleven rows of plants, each 3 ft. by 3 ft., giving a total area of just under half an acre. The experiment was on a moderate slope, the south corner being the highest. This corner was also the poorest with regard to depth of soil, and the soil in this part became very hard in dry periods.

Four seed treatments were used:

A. Untreated seed.

B. Seed dusted with "Granosan" (supplied by the Dupont Corporation, U.S.A.).

C. Seed delinted for 40 min. with concentrated sulphuric acid, washed, and then sterilised by immersion in 1 per cent. mercuric chloride solution under a partial vacuum for 15 min. and washed again. By this treatment it was hoped completely to avoid infection from *B. malvacearum* carried on the seed coat.

D. Seed soaked in a strong suspension of *B. malvacearum*.

1 B	2 D	3 C	4 A
5 C	6 A	7 D	8 B
9 D	10 B	11 A	12 C
13 A	14 C	15 B	16 D

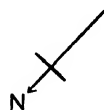


Fig. 1. Lay-out of experiment. Treatments: A. Control, untreated. B. Dusted with "Granosan." C. Delinted and sterilised. D. Inoculated.

406 *Angular Leaf-spot Disease of Cotton in Uganda*

The whole area was sown on August 5th after a few days' rain, but unfortunately no further rain fell for a fortnight, so that germination was slow and irregular. After germination the seedlings were thinned to two per hole, until the unfolding of the first two true leaves, when the plants were singled. Owing to the delayed germination this final thinning was not completed until towards the end of September. The first flowers appeared on the plot about October 20th, and the first picking took place on December 27th, the final picking being made on February 8th.

Observations on disease were made every 2 days up to September 21st, then twice weekly, and towards the end of the season at longer intervals. Each individual plant was examined for cotyledon, leaf, or stem infection and a plan made for each observation of the experiment to show the situation of the infection in the plots. Up to the time of singling the examination was made on a hole basis, that is, a single seedling in one hole showing infection was recorded as an infected hole. The three types of attack, cotyledon, leaf-spot, and stem lesion, were recorded separately as primary, secondary and black-arm infections.

Throughout the experiment careful meteorological records on the site were kept. These included observations of dry- and wet-bulb temperatures at 8.0 a.m. and at 2.0 p.m. local time, maximum and minimum temperatures, rainfall, and evaporation as measured by a Piché evaporimeter. In addition the full official records from Kololo Hill Observatory near Kampala were available.

The crop from each plot was collected in separate bags and weighed after the final picking. The number of bolls picked from each plant was recorded on a plan similar to those used for the infection records, no account being taken of boll diseases and pests. In future work it is hoped to elaborate the crop records in respect of such matters as the ratio of healthy to diseased bolls, clean to stained cotton, and so on. In the present case, however, it was deemed advisable to simplify crop records as much as possible, and to eliminate factors other than the disease under investigation. At Serere the lay-out of the experiment was similar to that at Kampala, but only the final yield figures and the number of plants are considered in this paper.

II. COURSE OF THE DISEASE.

(1) *Primary infection.* Fig. 2 shows the course of development of the primary infection at Kampala. Figs. 2, 3, and 4 have been prepared from the original dot-plans showing the location of the infected plants at each observation, and represent the distribution of the disease at

weekly intervals for the first half of the season, and at longer intervals later. As was to be expected, the primary infection was largely confined to the inoculated plots, although a few cases occurred on the control (untreated) areas. The seed was derived from a not very heavily attacked crop and so would not be expected to carry much natural infection. This

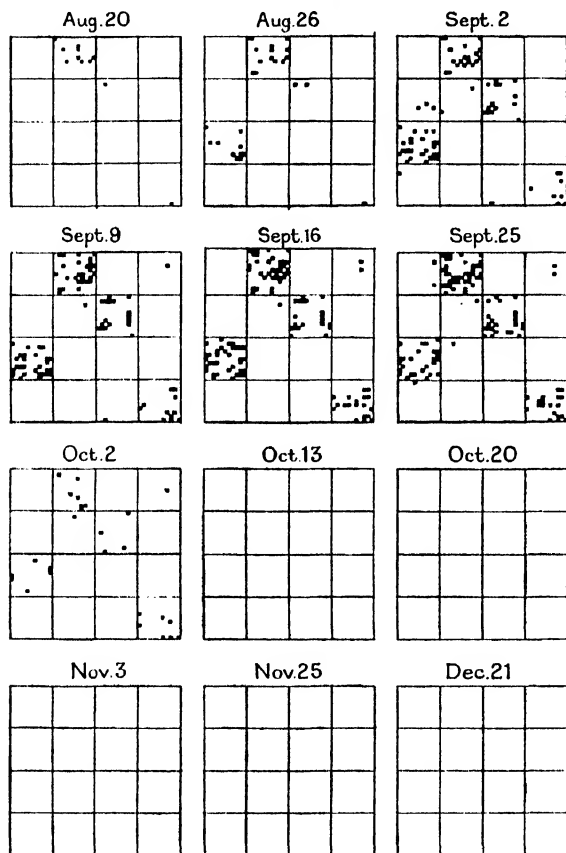


Fig. 2. Distribution of primary infection on different dates.

phase of the disease reached its maximum about the third week in September, 26 per cent. of the plants on the four inoculated plots showing infection on September 25th. The cotyledons were shed at this time, and the recorded primary infection decreased rapidly, becoming zero on October 13th when all cotyledons had fallen. The course of the primary infection on the inoculated plots is shown graphically in curve V of Fig. 6, as the mean percentage infection for the four plots of the treat-

408 *Angular Leaf-spot Disease of Cotton in Uganda*

ment at each date. Fig. 2 shows that no spread from plot to plot occurred during this phase of the disease. In view of the small size of the plants at this stage and their relatively large distance from one another, it would not be expected that such spread would occur.

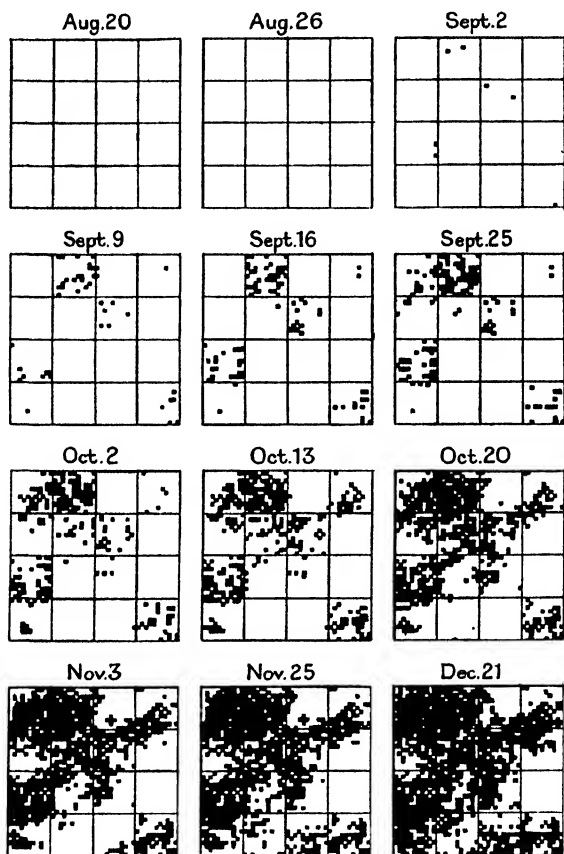


Fig. 3. Distribution of secondary (leaf-spot) infection.

(2) *Secondary infection.* The distribution and spread of the secondary infection is shown in Fig. 3, while the development is shown graphically in Fig. 6. Up to September 16th the secondary attack followed almost exactly the course of the primary infection. On that date there were only five plants outside the inoculated plots showing any infection, although on the latter 20 per cent. of the plants were attacked. Nine days later, however, on September 25th, infection had begun on plots 1 and 5, and by October 2nd these plots and also plots 6 and 13 were fairly

heavily attacked. Examination of the plan in Fig. 3 for these dates leaves little room for doubt that this infection spread in from the adjacent inoculated plots. The spread from the infected plots did not, however, occur in all directions equally. Clear examples of this are

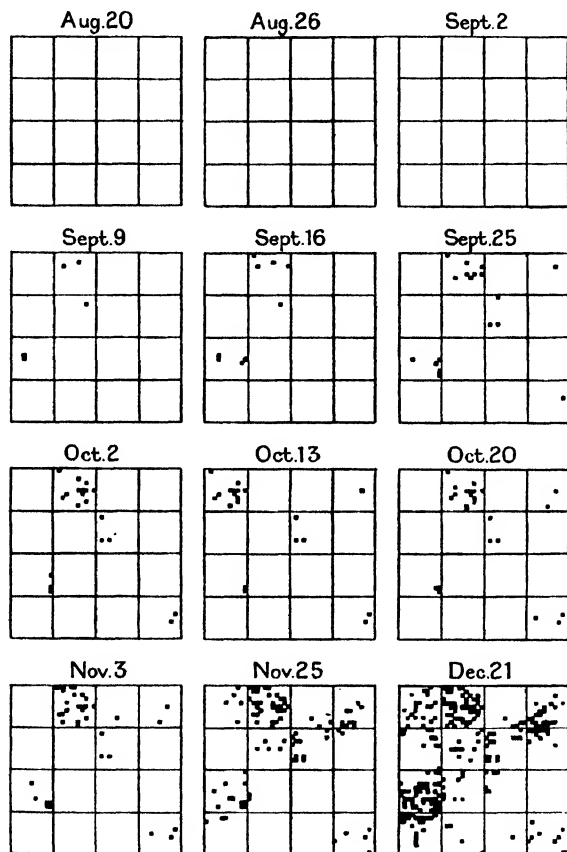


Fig. 4. Distribution of plants with black-arm infection.

given by plots 3 and 12. Plot 3 is bounded on the north-east by the very heavily infected plot 2, yet little spread occurred in this direction although plot 1 on the other side became severely attacked. Plot 12, again, is bounded to the north-west by plot 16, another inoculated area, but remained clean throughout the season. In general, there was a clear tendency for the spread to occur in a northerly direction, that is, down the slope of the land. This directional spread might be explained by one or more causes. An analysis of the prevailing winds, as far as the data

410 *Angular Leaf-spot Disease of Cotton in Uganda*

available allows, gives little indication of any decided preference for one direction, and we cannot definitely attribute the dissemination to this factor. Two other factors, both due to the slope of the ground, may be involved. In the first place, during a heavy rainstorm the splash droplets, which have been shown by Faulwetter⁽¹⁾ to consist of the water of the surface film on the leaf and thus to be the carrying agents of the disease, will tend on the whole to convey the infection down the slope rather than

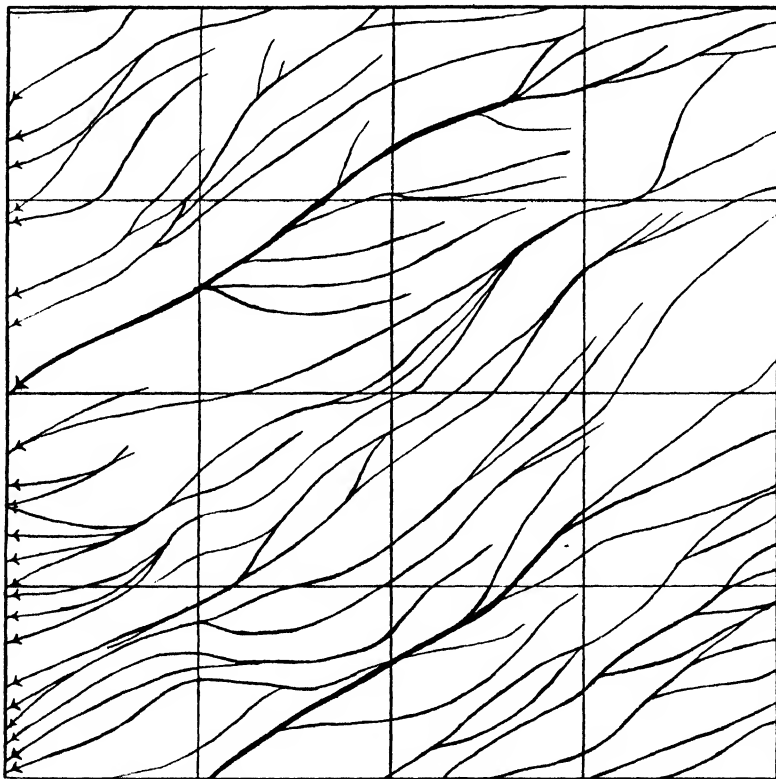


Fig. 5. Chart of main channels of surface-wash, October 5th.

up, in the absence of any strong counter-wind. In the second place, the infection might be carried in the water running over the surface of the ground and splashed up by falling rain. A chart of the main lines of surface wash is given in Fig. 5. This was made immediately after a very heavy fall of rain, and it will be seen that the direction of the lines follows very generally the direction of spread of the disease. This point is of interest in connection with the later discussion on the influence of meteorological conditions on the disease.

In spite, however, of the extensive spread of the disease, there remained a very marked difference in the degree of infection on the different plots. Curves I, II, III and IV of Fig. 6 show the course of the mean percentage infection for the four plots of each treatment. The eight

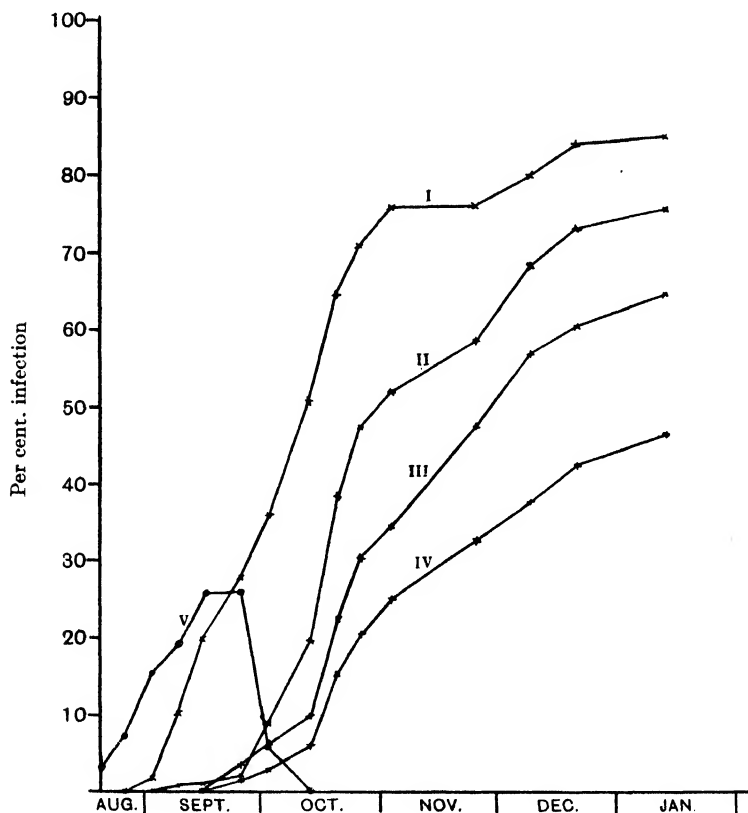


Fig. 6. Development of primary and secondary (leaf-spot) infection. Curve I, mean secondary infection treatment D (4 plots). Curve II, mean secondary infection treatment A (4 plots). Curve III, mean secondary infection treatment B (4 plots). Curve IV, mean secondary infection treatment C (4 plots). Curve V, mean primary infection treatment D (4 plots).

plots sown with treated seed show a marked reduction in the amount of disease, the delinted seed giving the healthiest crop. This difference in favour of the delinted seed is partly due to the complete freedom from attack of plot 12, a freedom which, as pointed out above, seems to be due largely to its position. The influence of the disease on the final yield will be discussed later in this paper.

412 *Angular Leaf-spot Disease of Cotton in Uganda*

(3) *Black-arm or stem infection.* Fig. 4 shows the distribution of plants having black-arm lesions, on the same dates as for the other phases of the disease, while the development of this phase is shown graphically in Fig. 7. The first point of note is that black-arm lesions were found only on plants previously recorded as showing angular leaf-spot. This manifestation of the disease occurs much later in the season than the leaf-spot type under Uganda conditions, although a few plants on the inoculated plots showed lesions quite early. It follows from the point noted that no independent spread of this phase of the disease was found, the attack being evenly distributed among the plants with leaf-spot. Arguing further from this it is clear that the amount of black-arm

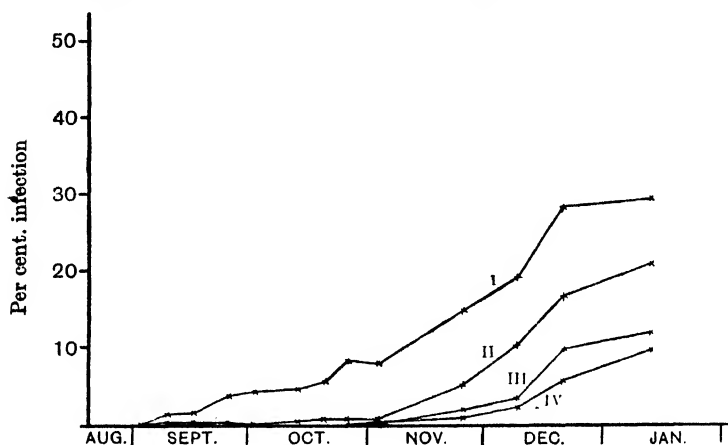


Fig. 7. Development of black-arm infection. Curve I, mean infection treatment D (4 plots). Curve II, mean infection treatment A (4 plots). Curve III, mean infection treatment B (4 plots). Curve IV, mean infection treatment C (4 plots).

attack on the plots of the different treatments would be expected to be in the same order as for the secondary attack; Fig. 7 shows that this was the case, the inoculated plots having the greatest infection and those sown with delinted seed the least. In view of the previous statements made by one of us (3) as to the origin of stem infections, an analysis of all infections on the stem and branches of the plants was made at the last four observations. A summary of the results is given in Table I. It will be seen that these figures bear out the theory that the majority of the lesions on stems and branches are extensions of lesions on the petioles. These petiolar infections, in turn, originate in the leaf, the organism spreading downwards through the cortical tissue of the leaf-stalk and reaching the cortex of the stem or branch. A point of interest

to note in the figures is the relatively small increase in the number of lesions on main stems in comparison with the increase in the number on branches and short shoots arising from secondary buds. This is in conformity with the rule that the organism can obtain a foothold only in young, immature tissues.

Table I.

Site and origin of black-arm lesions at four final examinations.

Treatments	Site					Origin			
	Stem	Branches	Short shoots	Boll wall	Boll pedicel	Petiole	Stipules	Bracts	Others
November 25th									
Untreated A	7	18	0	0	1	24	0	1	0
Dusted B	5	7	0	0	0	11	1	0	0
Delinted C	2	2	0	0	0	4	0	0	0
Inoculated D	49	39	1	0	0	84	1	0	3
December 9th									
Untreated A	10	52	14	0	3	74	2	3	0
Dusted B	5	18	4	1	3	26	1	3	0
Delinted C	2	8	0	3	1	8	1	1	0
Inoculated D	56	68	16	2	1	137	0	1	3
December 21st									
Untreated A	14	113	49	36	(6)	167	0	6	3
Dusted B	5	51	16	13	(5)	68	1	5	0
Delinted C	2	31	7	10	(1)	39	0	0	1
Inoculated D	58	139	61	39	4	249	3	4	6
January 16th									
Untreated A	14	167	76	31	(6)	248	0	6	3
Dusted B	6	79	48	12	(4)	128	1	5	0
Delinted C	2	44	17	15	1	63	0	0	1
Inoculated D	58	173	79	23	4	302	2	4	6

III. INFLUENCE OF METEOROLOGICAL CONDITIONS.

Fig. 8 shows the variations in the main meteorological conditions during the season, together with a curve showing the mean percentage secondary infection for the whole area. It is at once apparent that little or no definite information can be gleaned on the possible correlation between increase in disease and the main climatic factors. The variations in maximum and minimum temperatures were too small and too close together in point of time to be reflected in the disease curve, while the rainfall was too evenly spread over the growing season for any clearly marked correlation with the curve for disease to be evident. The latter shows two fairly well-pronounced rapid rises, the first between October 2nd and October 20th, and the second between November 25th and December 9th. While it is true that both of these rises follow periods of heavy rainfall about a week earlier, on the other hand the 3 days' heavy

414 *Angular Leaf-spot Disease of Cotton in Uganda*

rain of October 22nd-24th is not reflected in a subsequent increase of infection.

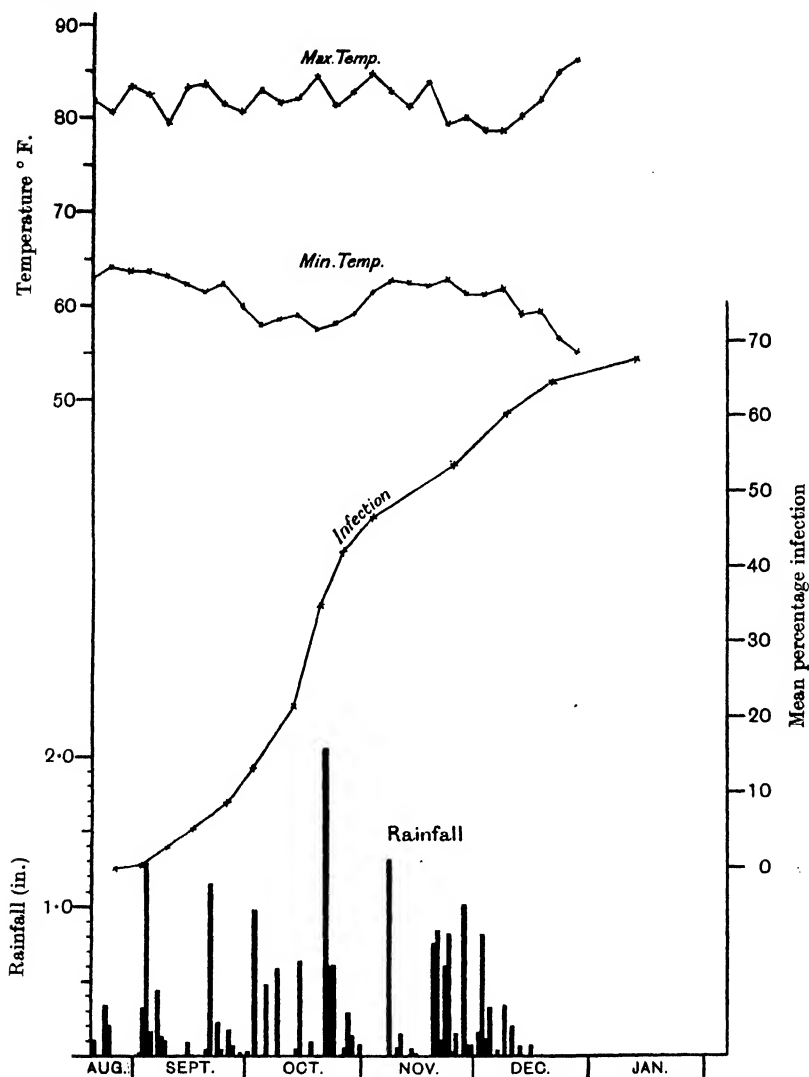


Fig. 8. Chart of rainfall (actual falls), maximum and minimum temperatures (smoothed 5-day means plotted each 5th day) and percentage secondary infection (mean of all plots).

It is clear, in any case, that a single experiment could not give reliable information on such a complex problem as the influence of climatic

conditions on disease. It is only by the accumulation of results over a number of years that an analysis of the separate factors becomes possible. It is hoped that the series of experiments, of which this is the first, will provide the necessary material for such an analysis. The general implications of the experiment and the suggestions that it gives for future modifications will be considered in the discussion at the end of this paper.

IV. STATISTICAL ANALYSIS OF RESULTS.

The two experiments, at Kampala and Serere, may best be considered together from the point of view of statistical analysis. The data provided by the Serere experiment is meagre, only the plant number, the weight of seed cotton, and the weight of the diseased bolls (which gave no cotton), being available for the individual plots. In the Kampala experiment the total yield of seed cotton, the plant number, and the number of "infection days" for angular leaf-spot and black-arm, are all available for the individual plots. These are given in Table II, and the corresponding figures for Serere in Table III. The number of infection days for each phase of the disease is obtained by computing the total number of days of infection of all infected plants, that is, the number of new infections at each observation multiplied by the number of days remaining to the end of the experiment. It should be emphasised, however, that the degree of replication (a single 4×4 Latin square at each centre)

Table II.

Yields and disease data of individual plots.

Kampala.

Plot	No of plants	No. of bolls	Total yield oz.	Infection days	
				Angular leaf-spot	Black-arm
1	113	378	48	8627	739
2	108	306	34	10951	3271
3	88	161	18	2123	215
4	83	167	17	3718	941
5	82	214	26	5610	332
6	99	224	25	7331	412
7	99	195	20	6751	995
8	108	200	23	2090	342
9	108	291	29	9512	2155
10	116	245	29	4840	263
11	103	238	28	3643	138
12	106	294	36	0	0
13	105	299	34	5214	740
14	101	286	32	1754	93
15	113	214	25	2641	23
16	99	152	19	5551	530

Table III.
Yields of individual plots.

*Serere.*¹

Plot	No. of plants	Yield of seed cotton oz.	Weight of diseased bolls lb.	Plot	No. of plants	Yield of seed cotton oz.	Weight of diseased bolls lb.
1	103	9.3	1.50	9	99	13.6	2.50
2	109	8.5	1.66	10	110	13.1	3.13
3	104	13.6	2.84	11	106	17.6	3.81
4	114	23.0	4.16	12	113	14.6	5.63
5	108	10.1	2.50	13	114	12.5	2.00
6	106	12.0	2.50	14	99	8.0	1.66
7	117	15.5	3.19	15	114	10.9	3.00
8	111	18.4	4.50	16	115	17.8	5.53

is scarcely sufficient to provide any very precise amount of information on such questions as the connection between yield and disease.

Table IV.

Totals of yield and plant number for the different treatments.

Kampala.

	Un-treated	Dusted	De-linted	Inoculated	Mean	Standard error
Plant No.	390	450	377	414	407.8	14.9
Yield (oz.)	104	125	112	102	110.8	16.7
Correction for plant No.	+ 16.7	- 39.7	+ 28.9	- 5.9	—	—
Corrected yield	120.7	85.3	140.9	96.1	110.8	9.93

Increase in yield on plots treated alike for each additional plant (b) = + 0.9402.

Serere.

	Un-treated	Dusted	De-linted	Inoculated	Mean	Standard error
Plant No.	424	455	442	419	435	7.16
Yield (oz.)	57.0	64.2	44.0	53.3	54.6	4.29

The totals of yield and plant number in the different treatments are given in Table IV. At neither centre does an analysis of variance(2) of the yields, unadjusted for plant number, give any significant results, the standard errors per plot having the high values of 15.1 and 15.7 per cent. respectively. In the analysis of plant number, however, given in Table V, the effect of treatments almost reaches the 5 per cent. level of significance at Kampala and passes it at Serere. This is due at both stations to the high number of plants on the plots with dusted seed.

¹ As the Serere results are not considered in detail, the key-plan of treatments at this station has not been included. The lay-out was similar to, but not identical with, that at Kampala.

Taken together the two experiments show a significant increase in plant number with the dusting of the seed, there being no significant differences between the other treatments. This result confirms claims previously advanced that dusting improves total germination.

Table V.
Analysis of variance of plant number.

<i>Kampala.</i>				
	Degrees of freedom	Sum of squares	Mean squares	<i>z</i>
Rows	3	345.19	115.06	—
Columns	3	106.19	35.40	—
Treatments: Dusted <i>v.</i> others	1	595.03	595.03	1.184
Others	2	176.17	88.08	0.229
Total	3	771.20	257.06	0.763
Error	6	334.37	55.73	—
Total	15	1556.94	—	—

<i>Serere.</i>				
	Degrees of freedom	Sum of squares	Mean squares	<i>z</i>
Rows	3	37.00	12.33	—
Columns	3	162.50	54.17	—
Treatments: Dusted <i>v.</i> others	1	133.33	133.33	1.170
Others	2	73.17	36.56	0.524
Total	3	206.50	58.83	0.840
Error	6	77.00	12.83	—
Total	15	483.00	—	—

z 15 % point, 3 D.F., 0.780; 1 D.F., 0.895.
1 % point, 3 D.F., 1.140; 1 D.F., 1.310.

A further analysis of yield was made at both stations by eliminating the effect of plant number by an analysis of covariance(2). The Kampala analysis is given in Table VI. At Serere there was no appreciable regression of yield on plant number, and the analysis is without interest. At Kampala, however, the regression of yield on plant number is undoubtedly significant, the regression coefficient having the very high value of + 0.9402, indicating an increase of yield of this amount for each additional plant above the mean number of plants, although the mean yield per plant over the whole experiment is only 0.271. This can only mean that plant number is in some way correlated with the fertility of the plots, so that plots with a higher plant number yield more *per plant* than those with a lower plant number receiving the same treatment. This is not in fact unreasonable, since the chief cause of loss of stand was due to drought when the plants were young, and plots non-resistant to

418 *Angular Leaf-spot Disease of Cotton in Uganda*

this drought might be expected to be less resistant to later droughts, and also to contain a greater proportion of damaged plants.

Table VI.

Analysis of covariance of yield and plant number at Kampala.

	Degrees of freedom	Sums of squares and products				Mean square
		Plant No. (<i>N</i>)	<i>N</i> × <i>Y</i>	Yield (<i>Y</i>)	<i>Y</i> · <i>bN</i>	
Rows	3	345·19	127·19	111·69	177·67	59·22
Columns	3	106·19	108·94	353·19	242·21	80·74
Treatments	3	771·19	157·19	81·69	467·84	155·95
Error	5	334·37	314·37	418·87	123·31	24·66
Total	14	1556·94	707·69	965·44	1011·03	

Since dusting has an effect on plant number the reduction of the yields of the different treatments to the basis of constant plant number, by means of the regression coefficient 0·9402, has no real validity. To make such a reduction valid it would have to be assumed that the high plant number of the dusted treatment was merely an indication that this treatment had fallen on a particularly favourable set of plots. Such an assumption is clearly unjustified. The reduction is shown in Table IV merely as an illustration of statistical procedure, and to emphasise the danger of an uncritical application of the methods of covariance to the elimination of plant number when the treatments themselves affect plant number. It will be noted that the yield from dusted seed, which was the highest, is now the lowest. The differences in corrected yield would be judged significant by the application of a *z* test to the mean square values of Table VI.

In order to see if there was any connection between yield and disease on similarly treated plots the partial regressions of the yield on the number of angular leaf-spot "infection days" and on the plant number were computed by the method of the analysis of covariance. The residual covariance between infection days and plant number happens to be zero (showing no connection between disease and plant number on plots treated alike), and the two partial regressions are therefore independent. The regression coefficient of yield on infection days is + 0·2591, showing an apparent positive correlation between yield and disease, but this correlation is by no means significant, since the amount of the sum of squares accounted for is only 31·45. There is thus no indication of any connection between yield and disease, as measured by the number of angular leaf-spot infection days. Similar results are obtained by taking the number of black-arm infection days as a measure of disease.

The analysis of the number of angular leaf-spot infection days shows

that the inoculated plants have a significantly higher, and the delinted plants a significantly lower, incidence of disease than the other two treatments. The lesser infection of the delinted as compared with the dusted plots may be in part a positional effect. These differences persist in the black-arm phase of the disease. The results are shown in Table VII.

Table VII.

Number of infection days.

	Untreated	Dusted	Delinted	Inoculated	S.E.
Angular leaf-spot	19906	18198	9487	32765	± 1770
Black-arm	2231	1367	640	6951	—

V. DISCUSSION.

It was pointed out in the introductory part of this paper that the experiment was of a preliminary nature, and was designed to investigate the possibilities of carrying out a field test of the spread of a disease and of its effect on the crop, which should be susceptible of statistical analysis. While it was not to be expected that this first experiment would lead to any definite conclusions, yet certain indications emerge of lines worth further study, and the analysis of the results offers suggestions for future modifications in the technique.

It is clear that the seed treatments employed have had a marked effect both on the germination of the seed as shown by the final stand obtained, and on the incidence of the disease. Treatment of the seed with a bactericidal dust resulted in a significant increase in the total germination, while sterilisation with sulphuric acid and mercuric chloride greatly reduced the amount of the disease in all its phases. It is perhaps unfortunate, from the point of view of seed treatment, that so little primary infection occurred on the untreated seed. On the other hand, the fact that this primary attack was practically limited to the inoculated plots gave better opportunity for observing directional spread. There can be no doubt that the spread of the disease has some relation to the lie of the land and the consequent surface wash. It was hoped at the inception of the work to obtain some information on the correlation between the incidence and spread of the disease and meteorological conditions, especially rainfall, but the distribution of the rainfall during the season was too uniform to give more than slight indications of its effect on the attack. In any case, experiments repeated over several years will be necessary before any exact analysis of the variable climatic factors can be made.

A further point on which it is hoped these experiments will give information is the effect of the disease on the final yield. Here the great

420 *Angular Leaf-spot Disease of Cotton in Uganda*

difficulty is to obtain a single figure for a plot which will give a measure of the degree of incidence of the disease. The final percentage infection at the time of picking is hardly a fair measure, since some plants may have been attacked very late in the season when the disease will have little effect on the final yield. The figure adopted of "infection days" would appear to give the fairest estimate, since, *cacteris paribus*, early infection persisting throughout the season is more likely to influence the yield than a late infection. The adoption of "infection days" as the measure gives due weight to the time factor in disease. The present experiment does not give any indication of a correlation between yield and disease. In future work it is proposed to separate the two investigations of the effect of disease on yield and of the influence of climatic factors on the disease, and two entirely different experiments to study these problems have been devised and are now being carried out in Uganda. It is hoped by divorcing the two distinct problems to obtain more precise information on each.

VI. SUMMARY.

Experiments on the incidence and spread of the angular leaf-spot disease of cotton, carried out at two centres in Uganda, are described.

Treatment of the seed by sterilisation with sulphuric acid and mercuric chloride resulted in a reduction in the amount of the disease throughout the season.

Treatment of the seed with a bactericidal dust had a significant effect on total germination, the plots sown with this seed having a greater number of plants at the end of the season, independently of those killed by the disease.

Primary infection was almost entirely limited to plots sown with seed inoculated with the organism.

Spread of the disease occurred in a direction down the slope of the ground and along the lines of surface wash.

The implications of the experiment are discussed and proposals made for modifications in technique.

REFERENCES.

- (1) FAULWETTER, R. C. (1917). Wind-blown rain, a factor in disease dissemination. *Journ. Agr. Res.* x, 639-48.
- (2) FISHER, R. A. (1932). *Statistical Methods for Research Workers*. Fourth Ed. London: Oliver and Boyd.
- (3) HANSFORD, C. G. (1931). *Rep. Dept. Agric. Uganda for year ended Dec. 31st, 1930*. Reprinted in *Empire Cotton Growing Rev.* ix, 21-31, 1932.

(Received January 19th, 1933.)

[FROM THE ANNALS OF APPLIED BIOLOGY, VOL. XX, No. 4,
pp. 590-611, NOVEMBER, 1933.]

[All rights reserved.]

THE INFLUENCE OF ENVIRONMENTAL CONDITIONS ON THE DEVELOPMENT OF THE ANGULAR LEAF-SPOT DISEASE OF COTTON

V. THE INFLUENCE OF ALTERNATING AND VARYING CONDITIONS ON INFECTION

By R. H. STOUGHTON, D.Sc.

(*Department of Plant Pathology, Rothamsted Experimental
Station, Harpenden.*)

INTRODUCTORY.

IN earlier papers of this series the influence of soil temperature⁽³⁾, air temperature⁽⁴⁾ and atmospheric humidity⁽⁵⁾, in conditioning the attack of young cotton plants by the bacterial parasite, *Bacterium malvacearum*, were considered. In all the experiments so far described the environmental factors under consideration were maintained constant throughout each experiment, in an attempt to disentangle the individual effects. In the experiments to be outlined in this paper the effect has been studied of varying one or more of the factors either in a uniform manner or by an abrupt transition from one value to another at different periods during the incubational period of the disease. In some of the experiments a regular diurnal variation has been arranged, and such conditions approximate more closely to those occurring in nature than has been the case in any of the experiments previously described. It is clear that experiments on the influence of varying conditions cannot be interpreted save in the light of similar tests carried out with the conditions maintained constant, but when the results of the latter are available the effect of fluctuating conditions may throw useful light on the problem of the influence of the environment in nature.

These experiments have all been carried out in the special Rothamsted control chambers erected and operated under a grant from the Empire Marketing Board⁽²⁾. The seed used throughout the work has been the "Sakellarides" variety from the Sudan, supplied by the courtesy of Mr R. E. Massey, Botanist to the Sudan Government.

THE INFLUENCE OF SOIL TEMPERATURE VARIATIONS.

Regular diurnal variation.

Exp. 1. In order to provide for an even diurnal variation of soil temperature some modifications in the standard controls of one chamber were necessary. The water from the soil temperature tank was drained out and the tank dried. A length of rubber tubing connected to an electric blower was passed through the water-inlet tube and brought down inside the tank, the open end being turned upwards and held in a retort clamp standing on the floor of the tank. When the soil tins were in place, the air blown into the tank escaped through the open water-outlet tube. The mercury thermostat was removed and the hole in the cover plugged. The relay for soil temperature control was removed from the electrical circuit to the tank heaters and in its place was substituted a small automatic time-switch. Two of the three heaters were disconnected, and the time-switch was set to cut in the supply to the remaining heater at 12 noon, and to cut out at 12 midnight. By adjustment of the air stream through the tank it was possible to obtain a gradual rise in the temperature of the soil in the tins for 12 hours, followed by a gradual fall to the original temperature. This fluctuation remained very regular and constant throughout the experiment, ranging from 25 to 37° C. Three of the remaining chambers were used with the standard controls, and the soil temperature thermostats were set to give temperatures of 25–26° C., 30–31° C., and 37–38° C., respectively. The air temperature thermostats in all four chambers were set for a constant temperature of 27° C. Air humidity was not controlled in these experiments, but exceeded 80 per cent. in all chambers except during short periods in the chamber arranged for the alternating soil temperature.

Before sowing, the seed was soaked in a strong emulsion of a virulent culture of *B. malvacearum*, and then sown at the rate of ten seeds per tin, giving a total of eighty seeds in each chamber. The seedlings appeared in 2 days at the higher temperature and in 3 to 4 at the lower. When the seedlings appeared above the soil, the artificial illumination was turned on, and the lighting control time-switch set for a lighting period of 14 hours, from 6 p.m. to 8 a.m. Thus the period of maximum soil temperature coincided with the time of illumination.

Infection spots on the cotyledons were visible very soon after germination. The spots were in most cases very small on the seedlings at the two higher soil temperatures, but were larger at the soil temperature of 25° C. The cotyledons were fully developed and infection appeared to be

complete 12 days after sowing, and the seedlings were then carefully examined for infection. The results are given in Table I, where the total number of seedlings in each tin, the number infected, the average germination and the average percentage infection are given.

Table I.

Infection at the four different soil temperatures. Exp. 1.

Tin	25-26° C.		30-31° C.		37-38° C.		Alternating 25-37° C.	
	No. of seedlings	No. in- fected	No. of seedlings	No. in- fected	No. of seedlings	No. in- fected	No. of seedlings	No. in- fected
I	7	0	6	2	4	1	8	1
II	7	4	8	2	7	0	7	1
III	7	1	8	1	7	1	6	1
IV	7	1	8	4	7	0	9	1
V	9	5	8	2	7	0	7	4
VI	9	4	8	1	5	2	6	2
VII	8	3	7	1	3	0	5	0
VIII	10	4	Accidentally destroyed		6	1	9	1
Total	64	22	53	13	46	5	57	11
%	80	34.4	76.4	22.6	57	10.9	71	19.3

It will be seen that the percentage infection decreases with increase of constant soil temperature, as was found in the earlier experiments on this factor (3). The amount of infection under the condition of a regular daily variation in soil temperature is somewhat less than at a constant temperature equal to the mean of the variation, *i.e.* 30-31° C., but the difference is hardly significant.

Exp. 2. This experiment was in all respects an exact repetition of the previous one, carried out under the same conditions. The results are given in Table II.

Table II.

Infection at the four different soil temperatures. Exp. 2.

Tin	25-26° C.		30-31° C.		37-38° C.		Alternating 25-37° C.	
	No. of seedlings	No. in- fected	No. of seedlings	No. in- fected	No. of seedlings	No. in- fected	No. of seedlings	No. in- fected
I	12	0	12	0	9	0	11	1
II	12	3	12	1	9	0	9	0
III	12	0	9	1	10	0	10	0
IV	11	3	11	1	10	0	11	0
V	12	3	12	2	10	0	10	1
VI	12	2	11	1	8	0	11	0
VII	12	1	12	2	8	0	11	2
VIII	11	1	12	0	10	0	11	0
Total	94	13	91	8	74	0	84	4
%	98	13.8	95	8.8	77	0	87	4.8

The general level of infection was lower than in Exp. 1, probably owing to a lesser degree of virulence of the culture used, but the order of the results is precisely as in the first experiment. It may be concluded, then, from these two trials that the effect of a regular diurnal variation of soil temperature on infection under these conditions is approximately equivalent to that of a constant temperature near to, or slightly above, the mean of the fluctuating temperature.

Abrupt change.

Having established the effect of a regular daily variation in soil temperature it remained to investigate the time factor in the influence of the temperature of the soil, that is to say, whether the temperature at the time of germination, or the temperature after germination has occurred, is the determining factor in controlling the amount of primary infection. To investigate this an experiment was carried out in which the soil temperature was altered at a definite time after sowing, and thereafter maintained constant at the changed temperature. At the same time an attempt was made to study the effect of soil moisture and of different types of soil on the primary infection. Two very different soils were used in this experiment. One was the Gezira cotton soil, used in all previous experiments, while the other was an old turf loam which had been used for tomato culture in the glasshouse.

Exp. 3. A sufficient quantity of each soil to fill twenty-four of the soil tins was prepared by spreading on a concrete floor to dry to a moderately low moisture content. Each batch of soil was then thoroughly mixed to ensure a uniform moisture content and twenty-four tins filled with each soil. Four tins of each soil were then placed in the control chambers, the tins being arranged alternately to allow for any unevenness in the distribution of light from the electric lamps. Two tins of each soil in each chamber were then watered with 1000 c.c. of tap water and two with 400 c.c. to give two different moisture contents. Each treatment was thus duplicated in each chamber. Samples of soil from each tin were taken after 2 days and the moisture contents determined. These gave average values of 26 and 23 per cent. for the "wet" and "dry" Gezira soils respectively, and 32 and 29.5 per cent. for the glasshouse soils. An amount of water was then added to each tin calculated to bring the moisture contents approximately to 30 and 23 per cent. for the Gezira, and 35 and 29.5 per cent. for the glasshouse soils. By this treatment the "wet" soils were nearly saturated and the "dry" were in a good condition for normal growth though rather on the dry side. After adjustment of the soil

temperatures to the values to be referred to later, the tins were sown at the rate of twenty seeds per tin with cotton seed from the normal Sudan crop of 1931-2. Before sowing, the seed was soaked in a very strong emulsion of *B. malvacearum* in water under the vacuum pump and then spread out overnight in the laboratory to become nearly dry.

The soil temperature in the chambers at the time of sowing and the subsequent changes were as follows:

Chamber 1.	20° C.,	constant.		
„	2.	20° C.,	raised to 33° C.	2 days after sowing.
„	3.	20° C.,	„	3 „
„	4.	33° C.,	constant.	
„	5.	33° C.,	reduced to 20° C.	2 days after sowing.
„	6.	33° C.,	„	3 „

The air temperature in all chambers was controlled at 32-33° C. The humidity was uncontrolled but was high in all chambers, though naturally lower in the chambers with a low soil temperature.

During growth, water was added from time to time in amounts thought to be necessary to maintain the moisture contents of the soils. This was, of course, largely a matter of guesswork, and in fact rather too much water was added, so that in most cases the moisture content rose during the experiment. At the close of the experiment the moisture content of each tin was again determined. The results of this determination are shown in Table III.

It will be seen that while the average values for the Gezira soils were maintained fairly well, the glasshouse soils were decidedly wetter at the end of the experiment than at the beginning. The differences between the "wet" and the "dry" soils was, however, retained throughout.

Germination was, unfortunately, poor, less than half the seeds sown producing plants. The experiment was allowed to run for two weeks, and at the end of this time the seedlings were examined for primary infection. The infection was not severe, but in accordance with the general rule for these experiments the presence of a single infection-spot on a cotyledon was counted as an infected plant. The results of the examination are shown in Table IV, which gives the total number of plants in each tin, the number infected, the percentage germination, and the percentage infection for each soil temperature condition and each type of soil.

Considering first the influence of soil temperature, a comparison of the total percentage infection in Chamber 1 (20° C., constant) with that in Chamber 4 (33° C., constant) shows the expected effect of reduction

Table III.
Moisture contents of tins of soil. Exp. 3.

Soil	Initial moisture content	Final moisture content							Mean %
		All chambers							
		Chamber 1	Chamber 2	Chamber 3	Chamber 4	Chamber 5	Chamber 6		
Gezira	"Wet" I	30	30.2	32.0	33.2	27.9	31.4	31.7	30.6
	" II	30	29.8	28.5	33.9	26.9	29.8	31.5	31.5
Glasshouse	" I	35	40.9	41.2	44.9	38.7	45.8	46.2	42.7
	" II	35	38.3	41.6	45.0	36.6	47.2	46.1	42.7
Gezira	"Dry" I	23	25.3	25.8	25.2	23.6	24.2	20.1	24.0
	" II	23	25.6	23.3	24.0	23.7	23.9	22.8	24.0
Glasshouse	" I	29.5	35.8	37.2	34.3	32.1	31.6	33.2	34.3
	" II	29.5	33.6	35.5	36.8	32.7	34.0	34.4	34.3

Table IV.

Infection at different soil temperature conditions and soil moistures. Exp. 3.

Soil	Chamber 1		Chamber 2		Chamber 3		Chamber 4		Chamber 5		Chamber 6		Mean germination %	Mean infection %	
	No. of plants	No. infected	No. of plants	No. infected	No. of plants	No. infected	No. of plants	No. infected	No. of plants	No. infected	No. of plants	No. infected			
Gezira	"Wet"	I	12	5	10	7	8	5	10	1	7	1	8	0	29.4
	"	II	8	3	5	2	5	2	6	0	13	2	7	1	
Glasshouse	"	I	7	3	8	1	6	1	10	1	15	1	9	0	19.6
	"	II	13	6	6	3	4	2	4	0	6	0	9	2	
Gezira	"Dry"	I	7	2	9	2	6	0	7	1	10	3	9	2	21.9
	"	II	7	3	10	3	10	2	5	0	6	1	10	2	
Glasshouse	"	I	10	0	10	0	10	1	8	0	11	2	9	1	14.8
	"	II	12	3	6	0	4	2	10	2	8	2	10	1	
Total			76	25	64	18	53	15	60	5	76	12	71	9	
Mean (o/o)			46.5	32.9	40.0	28.2	33.1	28.3	37.5	8.3	47.5	15.8	44.3	12.7	

in infection with high temperatures. The importance of the time at which the high temperature prevails is, however, clearly shown by a comparison of the figures for the other chambers. Where the germinating seeds were exposed to a low temperature for the first 2, or 3, days after sowing (Chambers 2 and 3) a subsequent raising of the temperature after this period has not appreciably reduced the amount of infection compared with those exposed to a low temperature throughout. On the other hand, a high temperature at the time of sowing and for the following 2, or 3, days (Chambers 5 and 6) reduces the infection very greatly, in spite of the subsequent reduction in temperature. The differences in infection between Chambers 4, 5 and 6 are of doubtful significance, although there are indications that the reduction in temperature 2 days after sowing has allowed some increase in infection over the controls to occur. The experiment shows clearly that the soil temperature is of chief importance at the time of sowing and during the first few days of germination, a high temperature at this period causing a considerable reduction in the amount of infection, while subsequent variations of temperature exert little or no effect.

Turning now to the influence of type of soil and moisture content, the figures given in the last two columns of Table IV show some interesting results. In the first place it is apparent that neither the type of soil nor the moisture content have had any appreciable effect on the total germination. The seeds in the "wet" series germinated more quickly than those in the "dry," but the final numbers are approximately the same in all series. On the other hand, both soil type and moisture content appear to have exercised some influence on the amount of infection. The mean percentage infection for the Gezira soil is definitely greater than for the glasshouse loam, while within the soil type a high moisture content is associated with a higher degree of infection than occurs with a more normal amount of water. The differences observed in the latter case, namely 7 and 10 per cent., are not high, but the fact that they are in the same direction in both soils makes the assumption of a definite influence of moisture content of the soil a reasonable one. The argument is strengthened by an examination of the figures for the separate chambers, the highest infections of the whole experiment occurring in the "wet" Gezira soils at a low soil temperature (Chambers 1, 2 and 3), where the mean percentage infections for duplicates are 40, 60 and 54 per cent. respectively.

THE INFLUENCE OF AIR TEMPERATURE VARIATIONS.

Regular diurnal variation.

To provide for a regular daily variation of air temperature a special type of thermostat was designed and constructed by Messrs Venner Time Switches, Ltd., of London. Briefly, this instrument consists of a direct-acting thermostat controlling the temperature, while the setting of the instrument is continually altered by a circular cam, driven by a clock, and revolving once in each 24 hours. The cam can be designed to give any desired range of temperature, while provision is made for setting the instrument to any required mean temperature. In the instrument used the cam was designed to give a range of 10° C. on either side of the mean. This apparatus replaced the standard thermostat and relay in the electrical circuit to the air-chamber heating coils.

Exp. 4. The results of part of this experiment were given in an earlier paper of this series (4), which dealt with the effect of constant air temperatures, but they must be recapitulated here for comparison with the remainder of the experiment, which concerned the influence of alternating conditions. The forty-eight tins for the six chambers were filled with cotton soil imported from the Gezira, and sown, in the glasshouse, with four seeds per tin. When of a suitable size the plants were transferred to the chambers for a few days and then sprayed with a strong emulsion of the bacteria. Five of the chambers were arranged to give constant air temperatures of 39, 35, 30, 25, 23.5° C. respectively, while the sixth was provided with the special alternating thermostat set to give a daily range from 20 – 40° C. Owing to the heating effect of the soil, which was kept at a constant temperature of 27 – 28° C. in all chambers, the minimum temperature reached each day in this chamber was about 22° C., while the maximum attained was usually about 39° C. Thus the alternation covered the full range of temperatures provided in the other five chambers. The relative humidity in all chambers was controlled between 80–85 per cent. Half of the total number of plants in each chamber were sprayed in the dark and half in the light. Artificial illumination was provided for 16 hours daily, the times of illumination coinciding with the high temperature periods in the alternating temperature chamber. The approximate incubation periods for the disease at the different temperatures, that is, the time from inoculation to the appearance of visible symptoms, are given in Table V. The plants at 39° C. constant temperature made no growth and many were dying at the close of the experiment. No definite

infection was detectable on the leaves of these plants, and they are omitted in the further discussion.

Table V.

Incubation periods at the different air temperatures. Exp. 4.

Temperature	35° C.	30° C.	25° C.	23.5° C.	Alternating 22–39° C.
Incubation period (days)	6	7–8	12	14	11–12

The plants at 35° C. were examined after 21 days and the remainder 3 days later. The method adopted for estimating the incidence and degree of infection was considered in detail in an earlier paper (4). Each leaf was examined, the number of spots counted on the standard basis, and the results grouped into four classes: Class I, severe infection, fifty spots or more per leaf; Class II, moderate infection, twenty-five spots or more; Class III, light infection, ten spots or more; Class IV, very light infection, less than ten spots. The results are summarised in Table VI.

The figures relating to constant temperatures were considered in the previous paper, and we are concerned here only with the influence of the alternating temperature. As in the case of a regular diurnal variation of soil temperature, the effect of a similarly fluctuating air temperature is to result in a degree of infection approximately equivalent to that obtained at a constant temperature near to the mean of the alternation. The infection is somewhat less than at a constant temperature of 30° C. but decidedly higher than at 25° C. Consideration of the effect of light and time of spraying in relation to the period of illumination will be deferred until later in this paper. A second trial of the influence of a regular alternation of temperature was incorporated in the experiment next to be described, which concerned mainly the effect of abrupt changes in the air temperature during the incubation period.

THE INFLUENCE OF ABRUPT CHANGES IN AIR TEMPERATURE.

Exp. 5. This experiment was designed to test whether the determining factor in infection with regard to air temperature is the actual temperature at the time of inoculation or the mean temperature prevailing during the incubation period.

Gezira soil was used, and plants were raised in the soil tins in the glass-house. When the plants were about six weeks old they were transferred to the chambers for a few days before spraying. The soil temperature thermostats were set for a temperature of 30° C. and the humidity controls for a relative humidity of 85–90 per cent. The following plan of air temperature changes was carried out.

Table VI.
Distribution of infection in four classes at various air temperatures. Exp. 4.

	35° C.				30° C.				25° C.				23-5° C.				Alternating 22-39° C.			
	Dark		Light		Dark		Light		Dark		Light		Dark		Light		Dark		Light	
	No. of leaves	%	No. of leaves	%	No. of leaves	%	No. of leaves	%	No. of leaves	%	No. of leaves	%	No. of leaves	%	No. of leaves	%	No. of leaves	%	No. of leaves	%
Total no. of leaves ...	78	—	78	—	68	—	62	—	59	—	55	—	64	—	60	—	58	—	63	—
Class I, 50 spots and over	16	20.5	1	1.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1.6
" II, 25 "	3	3.9	1	1.3	2	2.9	1	1.6	0	0	0	0	0	0	0	0	0	0	2	3.2
" III, 10 "	7	8.7	2	2.6	6	8.8	3	4.8	1	1.7	0	0	0	0	0	0	0	1	1.7	0
" IV, less than 10 spots	26	33.4	16	20.5	23	33.9	11	17.8	5	8.5	4	7.3	4	6.3	3	5.0	9	15.5	12	19.1
Total no. of leaves infected	52	66.5	20	25.7	31	45.6	15	24.2	6	10.2	4	7.3	4	6.3	3	5.0	10	17.2	15	23.9
Av. no. of spots per leaf	18.70		2.32		4.71		1.60		0.61		0.18		0.17		0.07		0.78		2.13	

Table VII.
Distribution of infection in four classes at the various air temperature conditions. Exp. 5.

	Chamber 1		Chamber 2		Chamber 3		Chamber 4		Chamber 5		Chamber 6	
	No. of leaves	%	No. of leaves	%	No. of leaves	%	No. of leaves	%	No. of leaves	%	No. of leaves	%
Total no. of leaves ...	142	—	98	—	132	—	154	—	129	—	178	—
Class I, 50 spots and over	1	0.7	0	0.0	6	4.5	7	4.5	3	2.3	18	10.1
" II, 25 "	1	0.7	2	2.0	1	0.8	7	4.5	2	1.6	10	5.6
" III, 10 "	6	4.2	5	5.1	15	11.4	9	5.8	10	7.8	11	6.2
" IV, less than 10 spots	27	19.0	9	9.2	18	13.6	18	11.7	32	24.8	38	21.4
Total no. of leaves infected	35	24.6	16	16.3	40	30.3	41	26.5	47	36.5	77	43.3
Av. no. of spots per leaf	2.05		1.71		5.48		6.01		4.05		10.62	

Chamber 1. Temperature at time of spraying 34°C ., maintained constant for 24 hours, then reduced to 22°C . and thereafter kept at this temperature.

Chamber 2. As Chamber 1, but 34°C . maintained for 48 hours after spraying.

Chamber 3. Temperature at time of spraying 22°C ., maintained constant for 24 hours, then raised to 34°C . and thereafter kept at this temperature.

Chamber 4. As Chamber 3, but 22°C . maintained for 48 hours after spraying.

Chamber 5. Regular diurnal alternation $22\text{--}35^{\circ}\text{C}$.

Chamber 6. Constant temperature of $34\text{--}35^{\circ}\text{C}$.

As has always been the case in working at comparatively low air temperatures it proved impossible to prevent a small rise in air temperature when the lights were on, and where the figure of 22°C . is given it should be noted that this represents the mean temperature during the 24 hours, the maximum range being 4°C ., *i.e.* from 20 to 24°C . This difficulty did not arise with the higher temperature, and here the total range of variation did not exceed 1°C .

Infection was visible in 5–6 days in Chambers 5 and 6, in 6–7 days in Chambers 3 and 4, and in 7–8 days in Chambers 1 and 2. The plants were examined and the degree of infection estimated the following number of days after spraying: Chamber 6, 14 days, Chambers 5 and 3, 15 days, Chamber 4, 16 days, and Chambers 1 and 2, 17 days. The amount and severity of infection was estimated by the standard method and the results are summarised in Table VII.

As was to be expected, the greatest amount of infection occurred in Chamber 6 at a constant temperature of $34\text{--}35^{\circ}\text{C}$., and these plants also showed the greatest number of heavily infected leaves, *i.e.* Classes I and II. The plants sprayed at a lower and subsequently exposed to a higher temperature, Chambers 3 and 4, showed considerable reduction in the severity of infection, but the degree of infection as judged by the more severe classes still remained fairly high. The difference between these two chambers is not significant and one must conclude that an additional 24 hours at a low temperature after spraying had no appreciable effect on the ultimate infection. The plants sprayed at a high temperature which was then reduced, *i.e.* Chambers 1 and 2, showed a great decrease in the amount and severity of infection. Here again there is no significant difference between the two sets of plants. The plants in Chamber 5 which

were exposed to a regular diurnal variation of temperature showed an amount of infection less than those in Chambers 3 and 4, but considerably more than those in Chambers 1 and 2; that is to say, a degree of infection corresponding approximately to the amount to be expected at a constant temperature near to the mean of the variation.

One further experiment was carried out using the alternating temperature thermostat. This experiment also included certain effects of humidity variations which should more properly be considered in the next section, but as the chief results concern temperature changes they may be described here.

Exp. 6. Six-week old plants raised in the glasshouse in the usual manner were employed. These were transferred to the chambers and left for several days at a soil temperature of 30–32° C., and an air temperature of 34–35° C. The chambers were then arranged to give the following air conditions during the experiment:

Chamber 1. Air temperature 34–35° C., constant. Relative humidity 85–90 per cent.

Chamber 2. Air temperature 35° C., constant. Relative humidity 55 per cent. mean.

Chamber 3. As Chamber 2, but plants sprayed once daily with distilled water, commencing 24 hours after spraying. (The humidity was not allowed to rise as a result of this operation.)

Chamber 4. Air temperature 25° C., raised to 35° C. 24 hours after spraying and thereafter maintained constant. Relative humidity 85–90 per cent.

Chamber 5. Air temperature 35° C., but allowed to cool each day for 3 hours during non-illuminated period, beginning 14 hours after spraying. Relative humidity 85–90 per cent., but rose to saturation during each cooling period.

Chamber 6. Air temperature alternating, 23–40° C. Relative humidity 85–90 per cent.

All plants were sprayed with a strong suspension of a young culture of *B. malvacearum* at 6 p.m., that is, one hour after the lights were turned on. Infection developed rapidly, and the plants were examined 9 days later, and the incidence and severity of the infection estimated in the usual manner. The results are summarised in Table VIII.

So far as temperature changes are involved, we are concerned here only with the results from Chambers 1, 4, 5, and 6, in which the humidity was in all cases high, exceeding 85 per cent. Chamber 4, it will be noted,

Table VIII.

Distribution of infection in four classes at the various air temperature and humidity conditions. Exp. 6.

	Chamber 1		Chamber 2		Chamber 3		Chamber 4		Chamber 5		Chamber 6	
	No. of leaves	%	No. of leaves	%	No. of leaves	%	No. of leaves	%	No. of leaves	%	No. of leaves	%
Total no. of leaves ...	159	—	170	—	167	—	170	—	145	—	160	—
Class I, 50 spots and over	6	3.8	0	0.0	1	0.6	6	3.5	11	7.6	9	5.6
" II, 25 "	5	3.1	2	1.2	1	0.6	8	4.7	4	2.8	7	4.4
" III, 10 "	12	7.6	7	4.1	4	2.4	11	6.5	13	9.0	10	6.2
" IV, less than 10 spots	36	22.6	31	18.2	30	18.0	46	27.0	26	17.9	24	15.0
Total no. of leaves infected	59	37.1	40	23.5	36	21.6	71	41.7	54	37.3	50	31.2
Av. no. of spots per leaf	5.66		1.81		1.26		6.21		8.04		6.8	

Table IX.

Distribution of infection in four classes under different conditions of atmospheric humidity. Exp. 7.

	Chamber 1		Chamber 2		Chamber 3		Chamber 4		Chamber 5		Chamber 6	
	No. of leaves	%	No. of leaves	%	No. of leaves	%	No. of leaves	%	No. of leaves	%	No. of leaves	%
Total no. of leaves ...	186	—	159	—	182	—	185	—	174	—	174	—
Class I, 50 spots and over	24	12.9	2	1.3	12	6.6	8	4.3	5	2.9	1	0.6
" II, 25 "	5	2.6	4	2.5	6	3.3	10	5.4	6	3.4	3	1.7
" III, 10 "	11	5.8	10	6.3	15	8.2	12	6.5	18	10.3	6	3.4
" IV, less than 10 spots	25	13.2	24	15.1	35	19.2	22	11.9	26	14.9	28	16.1
Total no. of leaves infected	65	34.5	40	25.2	68	37.3	52	28.1	55	31.5	38	21.8
Av. no. of spots per leaf	11.5		3.07		7.50		6.05		5.25		1.92	

is a duplication of one of the tests carried out in Exp. 5 and it is clear that the results are similar. Comparing the figures for this chamber, where the plants were inoculated at a low temperature which was raised to 35° C. after 24 hours, with those for Chamber 1 (constant temperature of 34–35° C.), it is apparent that there is no significant difference in the amounts of infection; that is, a low temperature at the time of spraying does not influence the final degree of infection if the temperature rises soon after inoculation and remains high thereafter. Similarly, the results for Chamber 5 show that a sudden and brief cooling each day has little effect on infection. The differences between the figures for Chambers 1 and 5 are of doubtful significance, the increase in severe infections in Chamber 5 being offset by the decrease in moderate and very light infections. In the case of the alternating temperature (Chamber 6), the amount and distribution of infection is again not appreciably different from that found at 34–35° C. constant temperature. This is in accordance with the previous results, since in this experiment the maximum of the alternations was 40° C. and the mean approximately 32° C.

THE INFLUENCE OF AIR HUMIDITY VARIATIONS.

It was not found possible to arrange for a satisfactory regular diurnal variation in the humidity of the air within the chambers and the experiments to be described concern only the effect of abrupt changes in relative humidity. Throughout this work the measure used in expressing the moisture condition of the air has been "relative humidity," that is, the "percentage saturation" or the actual pressure of aqueous vapour expressed as a percentage of the saturation vapour pressure at the given temperature. It might be expected on *a priori* grounds that "saturation deficit," *i.e.* the numerical difference between the actual and the saturation vapour pressures at the given temperature, which gives a measure of the evaporating power of the air, would prove to be a better estimate of the air condition for disease studies. Conversion of the figures for relative humidity into saturation deficit in actual experiments in the past shows, however, that the former is more directly correlated with the results of disease estimations than the latter.

Exp. 7. Four to five young plants were raised in each tin, in the glass-house, and the tins transferred to the chambers at a soil temperature of 29° C. and an air temperature of 32° C. Artificial illumination was provided for the usual period of 16 hours daily. These conditions remained constant throughout the experiment. After a few days in the chambers

the plants were sprayed with a strong suspension of the bacteria and thereafter subjected to the following humidity conditions:

Chamber 1. 85-90 per cent. constant.

„	2.	50-51 per cent.	„	
„	3.	85-90 per cent. for 24 hours, thereafter	50-55 per cent.	
„	4.	85-90 per cent. for 48	„	50-55 per cent.
„	5.	50-55 per cent. for 24	„	85-90 per cent.
„	6.	50-55 per cent. for 48	„	85-90 per cent.

The disease developed rapidly, the first symptoms showing in 4 days, and the amount of infection was estimated 9 days after inoculation. The results are summarised in Table IX.

In accordance with earlier results obtained on the effect of constant humidities⁽⁵⁾ the plants in Chamber 2 (55 per cent. relative humidity constant) showed a very marked reduction in infection compared with those at the optimum conditions prevailing in Chamber 1 (85 per cent. relative humidity constant). The plants of Chambers 3 and 4 which were inoculated at a high humidity, lowered 24 and 48 hours later respectively, showed approximately the same amounts of infection. This infection, though less than in Chamber 1, was still considerable, showing that the effect of a high humidity at the time of inoculation is not cancelled by a subsequent drop in humidity. Considering now the effect of a low humidity at the time of inoculation it is apparent that provided the humidity becomes high soon after inoculation as in Chamber 5, where the plants were sprayed at 55 per cent. relative humidity raised to 85 per cent. 24 hours later, a fairly high degree of infection can still result. If, however, the humidity remains low for 2 days after inoculation (Chamber 6), then a subsequent rise in humidity does not increase the infection above that occurring in a constantly dry atmosphere.

Exp. 8. Another experiment was carried out as an exact repetition of that just described, except that only five chambers were in use, the test omitted being that corresponding to Chamber 4 in the last experiment; that is, humidity at time of inoculation 85 per cent. dropped to 55 per cent. 48 hours after spraying. Unfortunately, in this experiment, 3 days after inoculation, the main fuse blew on the circuit supplying the whole installation. This accident occurred during the night and as the control of all chambers was thus completely upset for several hours it was decided to remove the plants from the chambers and place them all in the glasshouse. For 3 days, therefore, the plants received the differential treatments and thereafter were all subjected to the same conditions.

The plants were examined for infection 14 days after inoculation and as the results were strikingly in accord with those of the previous experiment it is thought worth while to present a summary of them, in spite of the departure from the usual treatment. For the 3 days of differential treatment the soil temperatures were 29–30° C. and the air temperatures 33° C. in all chambers. The humidity conditions were as follows:

Chamber 1. 85 per cent., constant.

- | | | | |
|---|----|--|---|
| „ | 2. | 55 per cent. | „ |
| „ | 3. | 85 per cent. for 24 hours, 55 per cent. mean thereafter. | |
| „ | 4. | 55 per cent. for 24 hours, 85 per cent. | „ |
| „ | 5. | 55 per cent. for 48 hours, 85 per cent. | „ |

The results obtained are summarised in Table X.

As in the previous experiment, the plants sprayed under conditions of high humidity and subsequently exposed to a drier atmosphere (Chamber 3) still showed a high degree of infection in comparison with those at a constantly low humidity. The plants inoculated at a low humidity but exposed to wetter conditions within 24 hours (Chamber 4) again showed slightly more infection than those of Chamber 2, but this difference is of doubtful significance, except in so far as it runs parallel to that in the previous case. The plants of Chamber 5, which were exposed to the low humidity for 48 hours after spraying, showed the same amount of infection as those at a constant low humidity. The experiment is therefore of interest in adding further evidence in support of the hypothesis that the humidity of the air is only of consequence during the 2 days following inoculation. It is also interesting to note that the general level of infection is distinctly lower than in Exp. 7, which may be explained by the lower mean temperature to which the plants were exposed during the incubation period after removal to the glasshouse.

In the light of these experiments one may consider the results involving humidity differences given in the description of Exp. 6. It will be recalled that in this experiment one chamber (Chamber 2) was run at a low constant humidity, and another (Chamber 3) under similar conditions, except that the plants were sprayed once daily with distilled water without, however, allowing the general air humidity to rise. The results show that the infection in both these chambers was much lower than in any of the other cases, while the spraying did not increase the infection above the control. This is in accordance with the theory put forward in an earlier paper (5) that the importance of humidity lies in its control of the time during which the infection droplets persist. The low

Table X.

Distribution of infection in four classes under different conditions of atmospheric humidity. Exp. 8.

	Chamber 1		Chamber 2		Chamber 3		Chamber 4		Chamber 5	
	No. of leaves	%	No. of leaves	%	No. of leaves	%	No. of leaves	%	No. of leaves	%
Total no. of leaves ...	198	—	172	—	207	—	199	—	186	—
Class I, 50 spots and over	5	2.5	0	0.0	2	1.0	0	0.0	0	0.0
" II, 25 "	3	1.5	0	0.0	5	2.4	1	0.5	0	0.0
" III, 10 "	11	5.6	4	2.3	9	4.3	6	3.0	4	2.2
" IV, less than 10 spots	29	14.6	15	8.7	35	16.9	19	9.6	17	9.1
Total no. of leaves infected	48	24.2	19	11.0	51	24.6	26	13.1	21	11.3
Av. no. of spots per leaf	3.58		0.56		2.64		0.83		0.58	

Table XI.

Distribution of infection in four classes under different conditions of illumination. Exp. 9.

	35° C.				35° C. 17 hours' illumination				25° C. 17 hours' illumination				25° C. 17 hours' illumination			
	Con-tinuous darkness		Con-tinuous light		Dark		Light		Dark		Light		Dark		Light	
	No. of leaves	%	No. of leaves	%	No. of leaves	%	No. of leaves	%	No. of leaves	%	No. of leaves	%	No. of leaves	%	No. of leaves	%
Total no. of leaves ...	74	—	182	—	110	—	95	—	92	—	126	—	86	—	102	—
Class I, 50 spots and over	0	0.0	14	7.7	10	9.1	9	9.5	7	7.6	9	7.2	1	1.2	2	2.0
" II, 25 "	0	0.0	17	9.4	3	2.7	5	5.3	5	5.4	11	8.7	5	5.8	3	2.9
" III, 10 "	6	8.4	27	14.8	13	11.8	14	14.8	14	15.2	17	13.5	10	11.6	7	6.9
" IV, less than 10 spots	6	8.4	37	20.3	21	19.1	20	21.0	17	18.5	22	17.5	11	12.8	25	24.5
Total no. of leaves infected	12	16.8	95	52.2	47	42.7	48	50.6	43	46.7	59	46.9	27	31.4	37	36.3
Av. no. of spots per leaf	1.60		11.92		10.07		11.30		10.03		11.32		5.14		4.60	
													2.88		7.62	

humidity in Chamber 3 caused the fine droplets resulting from the spraying to dry almost immediately, so that no further opportunity for infection to take place was afforded by the treatment.

THE INFLUENCE OF LIGHT AND TIME OF INOCULATION.

It will be remembered that in *Exp. 4* a test was made of the influence of time of inoculation with reference to the illumination period on the subsequent infection. The results showed that, with the exception of the plants exposed to an alternating air temperature where the differences were small, the plants inoculated in the dark were, in every case, more heavily infected than those in the light. An experiment with a greater degree of replication was designed to test the truth of this result and also to investigate the general effect of light on infection.

Exp. 9. Plants were raised in the glasshouse in the usual manner in the special tins filled with Gezira soil. The experiment was begun late in the season, and the growth in the glasshouse was slow and somewhat irregular. For this reason the plants were transferred to the chambers for one week at 30° C. air temperature, 30° C. soil temperature, and 17 hours' illumination before inoculation. This resulted in good new growth. The treatments in the six chambers were then as follows:

Chamber 1. Air temperature 35° C. Continuous darkness, extraneous light excluded by sheeting air chamber with brown paper.

Chamber 2. Air temperature 35° C. Continuous illumination.

Chamber 3. Air temperature 35° C. 17 hours' illumination daily. Half total number of plants inoculated in the dark, half in the light.

Chamber 4. As Chamber 3.

Chamber 5. Air temperature 25° C., otherwise as Chamber 3.

Chamber 6. As Chamber 5.

Soil temperatures were maintained at 30° C., and air humidity at 85-90 per cent. relative saturation throughout. The plants receiving no illumination naturally failed to make any further growth, and became very sickly. They were accordingly examined for infection 8 days after inoculation, as any longer period would have resulted in the death of the majority. The remainder of the plants at 35° C. air temperature were examined 11 days, and those at 25° C., 14 days after inoculation. The results are summarised in the usual form in Table XI.

It is clear that plants kept in total darkness were highly resistant to attack, only very few spots being found on the leaves. The theory was suggested in an earlier paper⁽⁴⁾ that the degree of attack is correlated

with the carbohydrate content of the leaves, which is in turn conditioned by the temperature and the amount of illumination. In the case of the plants in total darkness, photosynthesis being absent, the available carbohydrates would be rapidly used up and the parasite would be unable to develop in the tissues. The remaining plants at 35° C. constant temperature showed no significant differences in infection whether exposed to continuous light or to 17 hours' illumination daily. Further than this, there was in this case no difference in infection between the plants inoculated in the dark and those in the light. The agreement between the duplicates is unusually close. With regard to the two chambers at 25° C., there is again no definite evidence of a significant difference due to time of inoculation. In Chamber 5 the agreement between the two half sets of plants is fairly close, and though in Chamber 6 there is a rather wide divergence in infection in the two heavier classes, this may have been in part due to the smaller number of leaves in the dark series, affording less opportunity for infection. It would appear, therefore, from this experiment, that the consistent differences in infection with time of inoculation found in Exp. 4 were due to some other factor, at present unknown, than the mere difference in time of spraying with regard to the period of illumination.

DISCUSSION.

The most striking fact which emerges from the experiments described in the present paper is that for two, at least, of the main environmental conditions, namely, soil temperature and air temperature, fluctuations in these factors do not appear to influence the incidence of the disease except in so far as they affect the mean value of the factor. That is to say, fluctuating temperatures, under the conditions prevailing in these experiments, have the same effect on the course of the disease as a constant temperature near to the mean of the variations. This fact is of considerable importance in field studies of the effect of climatic conditions on the epidemiology of the disease, since in all such work it is usually difficult to know whether to take the maximum, minimum or mean as the measure of the temperature conditions prevailing. The demonstration in these experiments that soil type and moisture content of the soil also affect the amount of primary infection under a given set of physical conditions is also of importance in field investigations, and may help to throw light on some of the obscure points in the geographical distribution of the disease.

A further point of note is the elucidation of the effect of the time-factor in variations of the environment. It has been shown by the experiments described that the time-factor in relation to air temperature and to air humidity is entirely different in the two cases. The actual value of the temperature at the time of inoculation is of little consequence, the important consideration being the mean value of the temperature during the incubation period, that is, during the 5 or 10 days (the time depending on the mean temperature) immediately following inoculation. In the case of air humidity the reverse is true, the humidity at the time of inoculation and for a period of 12 to 24 hours afterwards (the critical time again depending on the temperature) being the controlling factor, subsequent variations in humidity having little or no direct effect on the disease. These findings are fully in accordance with the theories put forward in the earlier papers, namely, that the importance of air temperature lies in its differential effect on the balance between the rates of metabolism of the parasite and of the host, while the action of humidity is in controlling the time during which the infection droplets persist.

The influence of soil temperature on primary infection appears to lie more in the direct action of temperature on the parasite, and the present experiments on the effect of abrupt changes in this factor lend further support to this hypothesis. The inhibiting effect of a high soil temperature at the time of sowing is not cancelled by a subsequent drop in temperature, provided the high temperature has prevailed for a sufficient time, about 2 days, to exert its action on the bacteria surrounding the germinating seed. More rapid variations produce only the same effect as a constant temperature near to the mean of the variations, as was pointed out previously.

The experiments described in the present paper conclude the series of investigations which it was proposed to carry out on the influence of environmental conditions on this disease. The method of approach to the problem has been to consider first the effect of a single factor kept at a series of constant values. This has been done for the three main meteorological conditions of soil temperature, air temperature and air humidity, and the results have been described in the earlier papers of the series. The influence of the various factors under constant conditions having been ascertained, it has then been possible to study the effect of changes in the factors during the course of the disease, all conditions save the one picked out for study being still maintained at a constant value, that is, to examine the action of the time-factor in variations of

the environment. Finally, more than one factor has been varied, and the results interpreted in the light of the previous conclusions. With the knowledge so obtained the problem may be transferred to the field, and experiments set up to determine how far the conclusions arrived at can be confirmed under natural conditions. It remains to be seen whether the results of these laboratory trials will be fully borne out by field studies such as those now in progress in Uganda⁽¹⁾.

SUMMARY.

1. Experiments on the influence of variations in the environmental conditions on the bacterial disease of cotton plants caused by *Bacterium malvacearum* are described.

2. A regular diurnal variation in soil temperature is shown to have the same effect on primary infection of seedlings as a constant temperature near the mean of the fluctuations.

3. The mean soil temperature at the time of sowing and for the first few days of germination is the chief controlling factor in primary infection, other factors being equal. Subsequent variations in the soil temperature have little effect on the incidence of the disease.

4. The amount of disease resulting from infection of the seed, *i.e.* primary infection, is higher at soil moisture contents approaching saturation than at normal moisture contents in a given type of soil.

5. The amount of primary infection at a given soil temperature and moisture content varies with the type of soil.

6. A regular diurnal variation of air temperature has the same effect on secondary infection resulting from spray-inoculation of young plants as a constant temperature near to, or slightly above, the mean of the variations.

7. Other things being equal, the amount of infection resulting from spray-inoculation depends upon the mean temperature prevailing during the incubation period of the disease, the actual temperature at the time of inoculation being unimportant.

8. Atmospheric humidity is a conditioning factor in secondary infection only during a short period (less than 48 hours) following inoculation. Its importance lies in its control of the time during which the infection 'droplets' persist. Once penetration of the tissues has been effected variations in the external humidity have little direct effect.

9. Plants kept in total darkness are relatively resistant to infection.

10. Plants grown in continuous light are no more susceptible than those grown under a daily period of 17 hours' illumination of approximately 1200 foot-candles.

11. The time of inoculation in relation to the period of illumination does not appear to have any marked effect on the amount of infection.

12. The relations of the whole series of experiments on the influence of environmental conditions are discussed.

REFERENCES.

- (1) HANSFORD, C. G., HOSKING, H. R., STOUGHTON, R. H. and YATES, F. (1933). An experiment on the incidence and spread of angular leaf-spot disease of cotton in Uganda. *Ann. Appl. Biol.* xx, 404-20.
- (2) STOUGHTON, R. H. (1930). Apparatus for the growing of plants in a controlled environment. *Ibid.* xvii, 90-106.
- (3) — (1930). The influence of environmental conditions on the development of the angular leaf-spot disease of cotton. II. The influence of soil temperature on primary and secondary infection of seedlings. *Ibid.* xvii, 493-503.
- (4) — (1931). The influence of environmental conditions on the development of the angular leaf-spot disease of cotton. III. The influence of air temperature on infection. *Ibid.* xviii, 524-34.
- (5) — (1932). The influence of environmental conditions on the development of the angular leaf-spot disease of cotton. IV. The influence of atmospheric humidity on infection. *Ibid.* xix, 370-7.

(Received April 7th, 1933.)

THE PHYSIOLOGY OF VIRUS DISEASES IN PLANTS

V. THE MOVEMENT OF THE VIRUS AGENT IN TOBACCO AND TOMATO

BY JOHN CALDWELL.

(Department of Plant Pathology, Rothamsted Experimental Station.)

(With Plate X.)

IN earlier papers various experiments were described which indicated that the virus agent is unable to enter unbroken cells and is unable to pass into or through dead tissue. This was interpreted to mean that the agent passes from cell to cell by the protoplasmic connections, and that protoplasmic continuity is necessary either (*a*) to carry the agent bodily from cell to cell, or (*b*) to transmit the toxin which gives rise to the virus symptoms.

It was shown that the virus of yellow mosaic of tomato moves freely about the plant, that it does not enter the water stream, and that when injected mechanically into the xylem vessels it is unable to leave them to enter the living cells. Some evidence was available also to show that the presence of phloem tissue proper is not necessary for the movement of the agent through the plant, and that movement can and does take place through living parenchymatous ground tissue. It was suggested that there was good reason for supposing that the virus agent travels from cell to cell along the protoplasmic strands, and that movement in the phloem takes place by virtue of the fact that the phloem is alive rather than by mass movement in which the virus is carried bodily along. These conclusions seemed to be borne out by the observations on the approximately equal rate of movement of virus both upwards and downwards in the plant. These observations had been made by Böning⁽³⁾ and by the writer.

The work of Bennett⁽¹⁾, on the other hand, with raspberry mosaic in raspberries tended to show that this virus travelled more definitely in the phloem, and "ringing" experiments seemed to indicate that the extra-cambial tissues were the paths of translocation in this case. It is possible, however, that ringing may have the effect of destroying the living tissues of the xylem and medullary regions.

McCubbin and Smith(10) and Böning(3) have measured the average rate of movement of the virus, and their data confirm the view, put forward in an earlier paper of this series, that the movement of the virus in tomato is not very rapid, even having regard to the fact that the virus, after inoculation into a leaf, does not appear to leave that leaf for some 48-72 hours.

Holmes(9), on the other hand, has shown that the virus of tobacco mosaic in tobacco spreads slowly for some days following inoculation, and that it moves rapidly thereafter along the veins and stems. Samuel(11) goes so far as to say "in an area closely corresponding to where the virus has spread starch formation is inhibited so that the path followed by the virus shows up as a light coloured area (after iodine treatment) in the treated leaves. An intimate relation between the vascular tissue and the path of travel of the virus is made strikingly evident by this treatment." In Holmes's experiments, while virus multiplication was found to occur in the inoculated leaf, or portion of leaf, little increase of the virus was found in the leaves of the other side, or in the opposite part of the lamina of leaf of which only a portion had been inoculated. The distribution of the virus was very variable and, in some portions of the plant, large quantities of virus were found after only a few days, while in others no virus was recoverable until many days had elapsed.

It seems, therefore, clear that the movement of the virus in the host is less simple than had previously been supposed, and this paper deals with some data which have been obtained in experiments designed to throw further light on the subject.

THE MOVEMENT AND MULTIPLICATION OF THE VIRUS.

One point must be considered before proceeding to the experimental work. No one, apparently, has so far dealt with the problem of whether the virus is multiplied in given portions of the plant, *e.g.* at the site of inoculation, and is thereafter transported bodily along the conducting elements as would be an elaborated food substance, or whether the virus multiplies as it moves along from cell to cell.

A determination of this point would throw considerable light on the nature of the virus. Holmes(9) seems to be of the opinion that the virus of tobacco mosaic in tobacco moves with the food substances. The earlier papers of the present series tend to the view that the virus was travelling, in tomato at any rate, independently of the movement of food. This would be in concord with the view that multiplication of the virus is coincident with movement, and, as a consequence, movement and multiplication

can take place only in the regions of actively metabolising cells. To establish definitely that multiplication and movement were coincident would be difficult—the technique of virus investigation is still very crude, but the data given below throw some light on the problem.

MATERIALS AND METHODS.

The virus agents used in this investigation were the aucuba or yellow mosaic of tomato and Johnson's No. 1 mosaic of tobacco. They were transmitted by juice inoculation in the hosts tomato—(*Lycopersicum esculentum*) var. "Kondine Red," and tobacco—*Nicotiana tabacum* var. "White Burley."

The juice was extracted in the manner described in the earlier papers and was usually filtered through fuller's earth before inoculation. This operation, which is easily carried out, apparently did not affect the virus agent and was satisfactory in removing from the macerated material the cell fragments and the chloroplasts which were present in it. Inoculations were made in every instance by rubbing leaves or parts of leaves with a cotton-wool swab soaked in the virus juice. This broke the epidermal hairs of the leaves without excessively damaging the mesophyll tissues. To assess the amount of virus present in the tissues, use was made of the plant *N. glutinosa*, which is particularly useful in this connection because the symptoms induced in the leaves by inoculation with either yellow mosaic of tomato or with tobacco mosaic are necrotic spots which are localised and which do not spread either over the rubbed leaf or from the rubbed leaf to others. Again, as has been shown by Holmes⁽⁸⁾ and in the papers of this series, the number of spots which appear after inoculation is an index of the amount of virus present in any given inoculum.

THE APPEARANCE OF VIRUS IN TISSUES OF INOCULATED PLANTS.

In an earlier paper⁽⁶⁾ it was noted that the symptoms of mosaic always appear on the developing leaves of the host plant, and it was suggested that this was due not primarily to the facts that the virus was not present elsewhere in the plant or induced chlorosis by the destruction of chloroplasts, but that the effect of the virus was to inhibit chlorophyll formation. This observation has been confirmed by Sheffield⁽¹²⁾ working in this laboratory, who has studied the cytology of the diseased plants. From these observations it has become clear that the appearance of symptoms is not a necessary consequence of the presence of virus, and that virus may have passed through or may even be present in tissues without

making obvious its presence. Again, the developing tissues in which symptoms appear, are also the most actively metabolising tissues and the most rapidly dividing, and the multiplication of virus seems to require rapid metabolism in the host cells. It does not follow, however, that the virus has not passed through other cells or even that the virus is not present in other cells in such small quantities as to make it difficult of demonstration. Experiments have shown that the virus can and does pass through tissues which do not show symptoms, and from which it is not possible to recover enough virus to demonstrate its presence.

In an earlier paper it was shown that the virus did travel through the tissues of a *Datura* plant and would cause symptoms in a tomato scion grafted on that plant, although the symptoms which appeared on the *Datura* stock were definitely localised and although no virus could be demonstrated in the tissues of the host plant, away from the place of inoculation. There is good evidence, therefore, for supposing that the absence of symptoms or even of the virus agent itself from any tissue is not necessarily proof that the virus has not at least passed through that tissue. Until the nature of the virus is more clearly comprehended, it is not possible to decide as to why some tissues should not retain the virus in them. Mention has been made of the fact that the chlorosis in mosaic diseases is due to an inhibition of the formation of chlorophyll by the tissues and that it, therefore, is most marked in the young and actively growing tissues. That the virus is also more abundant in the chlorotic leaves is easily demonstrated. Ten discs of tissue 5 mm. in diameter from the lower leaves of a tomato plant inoculated when it was fairly well grown were taken on April 28th. The plant had been inoculated on April 8th by rubbing the stem with virus juice. This ensured that the virus entered by the broken hairs without actually collecting on the surface of the leaves. The lower leaves of the plant were all free from symptoms of the disease although the upper leaves were chlorotic. The ten discs of tissue were macerated in 2 c.c. of water, and the whole was rubbed over the surface of some twelve leaves on two large plants of *Nicotiana glutinosa*. Similarly, ten discs of the yellow tissues of the upper leaves were taken and inoculated on the leaves of two other plants of *N. glutinosa*. The plants were kept in the glasshouse for a week, when it was found that the number of spots on the leaves inoculated with the yellow discs was much greater than that on the other leaves.

This result is probably not surprising, but that of another experiment of the same kind is more difficult of interpretation. There is evidence that leaves which were developing at the time of inoculation show, on maturity,

symptoms of mosaic at the basal end, and are normally green at the apical end. The reason for this is discussed later in this paper. The leaves which have developed since infection, on the other hand, are also not uniformly yellow. On the young leaves there are areas of green tissue which is indistinguishable from the normal green tissues of healthy plants. These areas are found irregularly over the leaves and are not associated with absence of growth after infection or with any recognisable difference in the tissues. Discs of green and of yellow tissues were taken and macerated with water. The macerated material was inoculated on to leaves of *N. glutinosa* in the usual way.

After a week it was found that the leaves inoculated with the green discs had many fewer spots than those inoculated with the yellow discs. The proportion was of the order of 1 to 5. It is, therefore, clear that the quantity of the virus in the green areas is much reduced.

The green areas in the tomato leaves are not very extensive, and it is probable that small portions of yellow tissues were taken with the green discs. Support is lent to this view by the observations made on tobacco mosaic in tobacco. One of the characteristic symptoms of Johnson's No. 1 mosaic in tobacco is the presence of dark green areas slightly raised on the yellowish green lamina of the affected leaves. These green areas are similar in colour to the healthy tissues, and are in marked contrast with the chlorotic leaf tissues. They are often sufficiently extensive to allow of the disc being removed without accidentally including some yellow tissue. Ten discs of this and of the chlorotic tissue were taken and inoculated, after maceration, on to leaves of *N. glutinosa*. About twenty times as many necrotic lesions developed on the leaves inoculated with the yellow discs as developed on the leaves inoculated with the green discs. These results indicated that the multiplication of the virus is not uniform throughout the tissues of the host plant, and that not only do adult tissues not induce much multiplication but that there is also some factor, at present not clearly comprehended, which makes certain irregular areas of the developing tissues unsuitable for multiplication of the virus. A knowledge of the factors which prevent this multiplication would probably be of immense service in elucidating the problem of the nature of the virus. The observation is of interest also in connection with the transmission of the virus through the seed. If comparatively large numbers of the cells of ordinary vegetative tissue, scattered at random among the mesophyll cells, are unsuitable for the development of the virus, it is possible that similar conditions may obtain in the floral parts. It is not disputed that the fruits of the tomato, for instance, or of tobacco contain large quantities

of virus but, even by those who hold that the embryos are affected, it has not been shown that more than a small proportion of the seeds do carry mosaic or streak. (See Berkeley and Madden (2).)

THE TRANSMISSION OF VIRUS THROUGH THE SEEDS OF TOMATO.

The question of the transmission of virus through the seeds of tomato has been for some time a vexed one. It has been variously held that the virus is, and that it is not, carried in the seed. Experiments of various kinds have been carried out, and some workers have shown that from 3-6 per cent. of the seeds of plants affected with streak or mosaic may carry the disease. Others have contended that the virus is never carried in the seed of the tomato. It is probable that experiments involving the inoculation of seedlings with crushed embryos from diseased plants may be discarded, because it is almost impossible to ensure that no trace of extra-embryonic tissue has been included in the inoculum. Since the fruits contain large quantities of virus the testa and integumentary tissues are necessarily infected. The experiments with growing seedlings are of more interest. The expression of symptoms of mosaic among seedlings is not different from that in older plants. Reference has before been made (5) to growing seedlings in cotton-wool impregnated with juice from aucuba diseased tomato leaves. When seeds are sown on such wool, or in soil watered with virus juice, germination is rapid, and the seedlings which develop are normal and are free from symptoms of disease. If, however, the rootlets or the cotyledons are damaged as they appear, symptoms appear on the young plants within a week and the chlorosis is found on all the leaves, including the first opened.

It is evident that there is no inherent cause for the non-development of symptoms in seedlings. If, therefore, symptoms do not appear at all or do appear only after the leaves have developed for some 2 or 3 weeks, there is reason for supposing that, in the first instance, the embryos did not contain virus, and in the second, there may have been secondary accidental infection. This does not preclude accidental infection taking place in the seedlings which show symptoms from the beginning—as in them infection may take place through damaged tissue rubbing on the testa. If this accidental infection did take place, since it would occur in the stage before the formation of the foliage leaf, the symptoms should appear on that and subsequent leaves.

In our glasshouses we raise some 8000 seedling tomatoes each year, and there has been no case of an indubitable seed infection in the last four years. This, of course, may be accounted for by the fact that seed

had been taken from healthy plants. To avoid this possibility, at various times seed has been sown from plants with marked symptoms of aucuba mosaic or of streak. The seeds have been sown in flat boxes with some 300 seeds in each and have been kept in warm insect-proof houses. In no instance in some thousands of seedlings has any case of virus disease been observed. Having regard to the extreme ease with which these viruses travel about the plant, it would appear that the ovular tissue is for some reason free from virus, since the rapidly growing embryo would appear to furnish the ideal tissue for the multiplication of the virus if it were present. Further evidence for the multiplication of the virus in embryonic and rapidly metabolising tissue is given below.

Similar experiments have been carried out with tobacco seed from plants infected with different virus diseases, and again in this plant no evidence of seed-borne infection was observed.

In Table I is given a list of the viruses which have been examined with a view to discovering if they are seed transmitted.

Table I.

The seed transmission of the virus.

Host	Virus	No. of seedlings infected
Tobacco	Hyoscyamus III*	None
"	Tobacco mosaic	"
"	Hyoscyamus III and "Green"	"
Tomato	Aucuba mosaic	"
"	Tobacco mosaic	"
"	"Streak"	"
"	"Spotted wilt"	"

* See M. A. Hamilton (7).

THE APPEARANCE OF VIRUS SYMPTOMS IN THE PLANTS.

Attention has been directed to the fact that virus symptoms appear in the growing tissues, and that the top portion of the plant is then the part in which the chlorosis is marked, if the plant were a fair size at the time of inoculation. A careful examination reveals the fact that among the leaves in the intermediate zone, that is, between those which are generally chlorotic and those in which no symptoms appear, there is a group of leaves—and this holds for both tomato and tobacco—in which the apical portion is apparently healthy and the basal portion chlorotic. The reason for this distribution of the symptoms becomes obvious when one remembers that the virus only inhibits chlorophyll formation and does not destroy preformed chloroplasts. The development of a leaf is most marked in the

basal region, and that portion is the last to stop growing. As a consequence, therefore, it is possible that if the apical region of a leaf had at the time of infection developed its chloroplasts, this basal portion would only be developing, and could therefore be liable to attack as a consequence of the virus infection. The development of a leaf is easily demonstrated by marking the lamina of a developing leaf with squares 1 cm. \times 1 cm. This was done for a developing tobacco leaf, and after a week a photograph of it was taken. It will be seen from the photograph in Plate X, fig. 1, that the marking was not geometrically accurate—the nature of the leaf of a tobacco does not allow of this—but that it is clear that the squares are much larger at the basal end than at the apical. At the same time, the Indian-ink lines are much less faint at the apical end of the leaf than at the basal. The growth, therefore, has been approximately twice as great at the basal end. It is, consequently, not surprising that the symptoms of virus disease tend to occur on that part of those leaves which were in the course of development at the time of inoculation.

The non-appearance of symptoms must not be taken as evidence of the absence of virus, but it does appear from the results which have been set out, that the virus increases very much more in tissues which are developing and which will, incidentally, show marked symptoms than in those tissues which have already developed.

THE MOVEMENT OF THE VIRUS FROM THE INFECTED LEAF.

Holmes has shown that the virus of tobacco mosaic moves in a peculiar way from the leaf where the inoculation was made. He attributes this to the fact that the virus travels with the food material, *i.e.* presumably in the phloem. The experiments now to be described were designed to examine this hypothesis. The first experiment was similar to those of Holmes. A large plant of tobacco some 18 in. high was selected, and inoculation was made by rubbing the inoculum on a small portion at the apical end of a mature leaf half-way up the plant. After 7 days the leaves were removed severally, and were crushed in a mortar with twice their own weight of water (the usual procedure for obtaining an inoculum). Each of the leaves was numbered. The side of the lamina on which the inoculation was made was numbered 1*a*, the opposite side 1*b*. The distance from the place of inoculation to the base of the leaf was 15 cm. The next higher leaf on the stem was numbered 2, the next 3, then 4, then 5, and the apical region of the stem, together with its leaves 6. The leaf next below the inoculated one was 2¹, the next lower 3¹, and the next again 4¹. Each of these inocula was rubbed on the leaves of plants of *N. glutinosa*,

which develops, with this virus, necrotic lesions. At the time of sampling the upper portion of the tobacco plant showed symptoms of mosaic. The results obtained on the fifth day after rubbing the *N. glutinosa* leaves are detailed in Table II.

Table II.

*Number of lesions on leaves of N. glutinosa inoculated
from the leaves of tobacco.*

Leaf 6 (including top of plant)	Many lesions
Leaf 5	Many lesions
Leaf 4	Very many lesions
Leaf 3	Many lesions
Leaf 2	No lesions
Petiole of infected leaf	Some lesions
Leaf 1a	Few lesions
Leaf 2 ¹	Very few lesions
Leaf 3 ¹	No lesions
Leaf 4 ¹	No lesions

This is not quite in accord with the data of Holmes, but the difference is, I think, attributable to the fact that the leaves on the opposite side of the plant in this experiment, viz. leaves 3 and 4, were not quite fully developed at the time of infection. In other words, the amount of virus present in a leaf at any given time depends not so much on whether the virus has reached that leaf as on whether, having reached it, multiplication has taken place. Leaf 2 illustrates this point; there is little reason inherently why the virus should not have reached leaf 2 when it has reached leaf 3, and no matter what the mechanism or path of movement may be, the virus must have passed within a maximum of a centimetre of leaf 2 to get to leaves 3 and 4, etc.

To discover if the absence of the virus from leaf 2 immediately above the infected one were due to the non-multiplication of the virus in the mature tissues of that leaf, a second experiment was set up. A plant, rather larger than the last was selected, and all the leaves with the exception of one half-way up the stem were removed. An inoculation was made on a small area of this leaf on one side of the lamina at the apical end. This area was 30 cm. from the base of the leaf. On the ninth days symptoms appeared on the leaves of the axillary buds which had developed in the interval. These axillary shoots corresponded to all the leaves on the plant, both above and below the treated petiole, and this would suggest that the presence of the embryonic tissues did induce the appearance of the virus in quantity in those regions where, when the adult leaves were present, it was in so small quantity as not to be demonstrable. Further evidence in support of this view is afforded by this additional observation.

The leaf on which the inoculation was made was divided into three parts: (1) the half-lamina on the same side as the inoculation, less the inoculated portion, (2) the other half-lamina, and (3) the mid-rib and the portion of the lamina immediately round it. The three portions were each macerated in water, and the inoculum rubbed on the leaves of plants of *N. glutinosa*. After a week these leaves were examined, when it was found that the lesions had developed only on those which had been inoculated with the material from the mid-rib.

The virus had multiplied in the region of the original inoculation where local symptoms appeared, and also in the mid-rib of the leaf. Multiplication had apparently not taken place in the other portions of the lamina. It is probable that the breaking of the cells set up some increase in the metabolism of the injured tissue, as one would expect, and that this accounts for the increase round the point of inoculation. Similarly there is probably more activity round the vascular tissue than in normal mesophyll cells and this, in turn, may account for the multiplication in the mid-rib. Alternatively, the movement down the mid-rib may be interpreted as indicating that the virus is being carried with the food material in the vascular tissue. This hypothesis is examined in the next section.

THE POSSIBILITY OF MOVEMENT OF THE VIRUS WITH THE FOOD MATERIAL.

It might well be argued that the fact that the older leaves do not develop virus might be due to the passage of all the food material downwards from the lamina into the main stem. Since the virus does not travel in the xylem⁽⁴⁾ but in the living tissue, the phloem might be the path by which movement took place, if that movement were due to the removal of the virus, passively, from place to place, with the food material. To clear up this point the following experiment was carried out. Some large tobacco plants were put in a warm, dark chamber for 2 days so as to allow of the removal of the reserve carbohydrates from the mesophyll tissues. The next day all the leaves but one, half-way up the stem, were removed. An inoculation was made with tobacco mosaic on an area of the remaining leaf in the same manner as before (see above), except that immediately after inoculation the leaf was completely darkened by being enclosed in a black cloth envelope which excluded the light. The effect of this treatment should be to reverse the normal movement of the food materials from this leaf. Since it is fully grown, it is presumable that the foodstuffs elaborated in it are sent down the petiole and are sent thereafter to the

embryonic tissues. Under this treatment, however, the other tissues of the plant should actually be elaborating more carbohydrate than are those of the inoculated leaf, and, consequently, the movement of the food material should be towards and not away from the treated leaf.

After an interval of 6 days symptoms appeared on the axillary shoots which had developed as a consequence of the mutilation. The leaves of all the axillary shoots developed symptoms, whether or not the inoculated leaf had been darkened. These tissues in which the symptoms appeared correspond exactly to the leaves of the plants in the earlier experiments, and this observation confirms the idea that a mature leaf may not induce the multiplication of the virus, whereas a rapidly growing one under the same conditions would. At the same time, the fact that darkening the inoculated leaf has no apparent effect on the infection of the plant, suggests that the amount of the virus is not dependent on that of the food supply.

This second point is confirmed by another experiment. Some large tobacco plants about a foot high were put in complete darkness for 48 hours. Thereafter they were inoculated with tobacco mosaic and returned to the light in a glasshouse. One set was inoculated on the uppermost leaf of inoculable size, the second set was inoculated on the third foliage leaf from the base and the third set was inoculated on the same leaf (the third from the base), this leaf being subsequently covered with a black cover which excluded the light. On the seventh day after inoculation all the plants showed symptoms of tobacco mosaic, there being not the slightest evidence that the virus in any one set had reached the apical region before that in the others.

A similar experiment was set up with tomatoes and aucuba mosaic. The tomato plants were about 9 in. high at the beginning of the experiment. They were kept for 48 hours in complete darkness. At the end of that period a small portion of the tissue of the stem above the axil of the second foliage leaf proper was removed. This had the effect of cutting off the direct vascular connection between this leaf and the upper portion of the plant. The appearance of the treated stem is indicated in Plate X, fig. 2. As a consequence of this it was expected that the axillary bud below the cut portion would grow immediately—as indeed it did.

The plants were then divided into three groups. The plants of the first group were inoculated with aucuba mosaic on the topmost leaf, which was sufficiently developed to allow of inoculation. The plants of the second group were inoculated on the distal pinna of the leaf below the notch in the stem. This was a fully mature leaf, and it was not expected that symptoms would appear on that leaf. The plants of the third group

were also inoculated on the second leaf above the cotyledons, and this leaf after inoculation was inserted into a black hood which excluded the light. The expectation was that the carbohydrates of this leaf had been completely used up, and that such carbohydrates as it obtained would be obtained from the rest of the plant. Here again, therefore, if there were a movement of food materials, it would be *into* the leaf and not out of it. Five days after inoculation all the plants, which were kept in a warm glasshouse, showed symptoms of aucuba mosaic on the top leaves. The axillary bud below the notch in the stem had developed in the interval, and its leaves also showed signs of mosaic in all cases. The symptoms appeared just as quickly on the plants with the darkened leaves as on the others. It appeared, therefore, that the virus had travelled up the plant from the lower rubbed leaves, and down the other plants from the upper rubbed leaves of the first and second groups respectively at approximately the same rate and that, despite the removal of the direct vascular connection of the lower axillary bud with the upper portion of the plant. At the same time the virus had multiplied in, and had moved out of, the darkened leaf and had moved about the plant apparently at the same rate as in the others, despite the fact that there could have been little, if any, movement of food material from that leaf. In all the plants, the lowermost leaves, including that in the axil of which the shoot grew out, showed no symptoms of virus, being at the time of infection fully mature. These experiments were repeated, use being made of the axillary shoots corresponding to the treated leaves. Similar results were obtained.

DISCUSSION.

Two main lines of work have been dealt with in this paper, viz. (1) the non-transmission of virus through the seed of tomato and tobacco, and (2) the movement of the virus as compared with that of the food materials in the plant.

The experiments with seedling inoculation show that there is no inherent reason why a young plant should not develop symptoms of virus. It has been found that, provided the tissues be broken, no matter how early infection be made, with either aucuba mosaic or "streak" virus, symptoms will appear on the first foliage leaf when it develops. It is, therefore, probable that virus which was in the embryo should induce symptoms in all the foliage leaves of the seedlings. If infection should take place by rupture of the hairs or tissues of the embryo and entry of the virus adhering to the integumentary tissue, again, symptoms would appear in the first-formed foliage leaf. In no instance, in the hundreds of

seedlings grown from seed taken from infected plants, has a single seedling been found in which, without actual inoculation, virus symptoms have developed. Inoculation in the usual way from those seedlings into other healthy seedlings has in no instance given evidence of the presence of the virus in a latent form.

The examination of the embryo in the early stages shows that growth of the embryonic tissue is very rapid, as also is the increase in size of the embryo. The difference in growth between the embryonic tissue and the normal ovular tissue would have the effect of breaking the protoplasmic connection between the embryo and the placental tissue so that the virus would normally have no opportunity of entering the embryo after development had begun. This would account for the absence of secondary infection of the embryo, but does not take account of the possibility of infection of the megaspore mother-cell at a much earlier stage. Reference has been made to the presence in the tissues of groups of cell which for some reason are apparently more resistant to the virus, or prove unsuitable for the multiplication of the virus within them. It may be that potential megaspore mother-cells are of this type. That the reproduction tissue proper may be unsuitable for the multiplication of virus is further borne out by the fact that I have been unable, so far, to demonstrate the transmission of the virus through the pollen. The amount of pollen which is available at any time from the flowers of virus diseased plants is not very large and the experiment, therefore, is not a highly critical one—the results being tentative rather than conclusive.

The experiment is further complicated by the fact that it is difficult to ensure that no pieces of tissue will be shaken off with the pollen grains and the occasional spots which do appear on the leaves of *N. glutinosa* after treatment are believed to be due to accidental infection, either from the surface of the pollen grains or from hairs, etc., which had broken off.

The procedure consisted of shaking the open flowers in a small glass dish into which the ripe pollen fell. This was subsequently macerated in water. As can be seen, there is considerable danger of other tissues being collected with the pollen, and so odd spots were discounted, it being felt that the thousands of pollen grains would, had they been infected, have supplied a great deal more virus than that found.

The results of the experiments on the movement of the virus in the plants indicate that the virus moves independently of the food materials. It would appear, therefore, that the virus is not carried mechanically from some source in which it has been elaborated, but rather that it moves outward in all directions from the place of infection, along the proto-

plasmic strands and increasing in amount as it passes from cell to cell. The mechanism by which this increase takes place is not yet understood.

SUMMARY.

Experiments on the movement of the virus of aucuba or yellow mosaic of the tomato in the host plant are described. It has been found that the presence of the virus in the tissues is not always associated with symptoms, and that the symptoms appear in those tissues which have developed after infection. It was also found that the distribution of the virus throughout the plant was not uniform. In the chlorotic tissues the virus content was higher than in the neighbouring green areas.

A large series of experiments has been carried out on the transmission of six different viruses in the seed of tomato or tobacco. In no instance was there any evidence of transmission, and it is suggested that the chance of seed transmission of these viruses is very slight.

The movement of the virus from an infected leaf and the possibility of its being carried with the food material were examined, and results are given which show that the virus can move independently of the food materials and that, under certain conditions, the virus apparently moves in the direction opposite to that of the metabolites.

This work was carried out under the auspices of the Empire Marketing Board.

REFERENCES.

- (1) BENNETT, C. W. (1927). Virus diseases of raspberries. *Mich. Exp. Stat. Bull.* No. 80.
- (2) BERKELEY, G. H. and MADDEN, G. O. (1932). Transmission of streak and mosaic diseases on tomato through seed. *Scient. Agric.* XIII, No. 3, pp. 194-7.
- (3) BÖNING, K. (1928). Beitrag zum Studium der Infektionsvorgänge pflanzlichen Viruskrankheiten. *Zeitschr. f. Parasitenkunde*, I, 198-230.
- (4) CALDWELL, J. (1930). The physiology of virus diseases in plants. I. The movement of aucuba mosaic in the tomato plant. *Ann. App. Biol.* XVIII, 279-98.
- (5) — (1932). The physiology of virus diseases in plants. III. Aucuba or yellow mosaic of tomato in *N. glutinosa* and other hosts. *Ibid.* XIX, 144-52.
- (6) — (1933). The physiology of virus diseases in plants. IV. The nature of the virus agent of aucuba or yellow mosaic of tomato. *Ibid.* XX, 100-15.
- (7) HAMILTON, M. A. (1932). On three new virus diseases of *Hyoscyamus Niger*. *Ibid.* XIX, 550-67.
- (8) HOLMES, F. O. (1929). Local lesions in tobacco mosaic. *Bot. Gaz.* LXXXVII, 39-55.
- (9) — (1932). Movement of mosaic virus from primary lesions in *Nicotiana tabacum*. Contributions from the *Boyce Thompson Institute*, IV, 297-323.

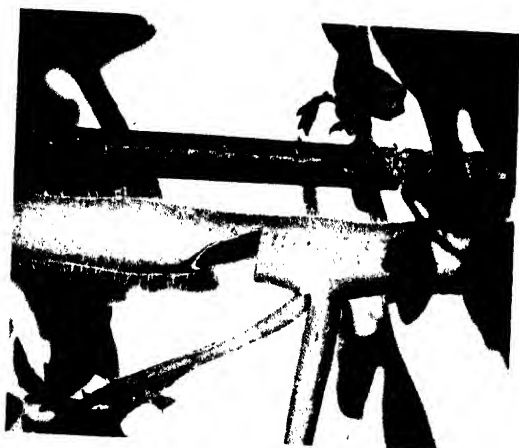


Fig. 2.

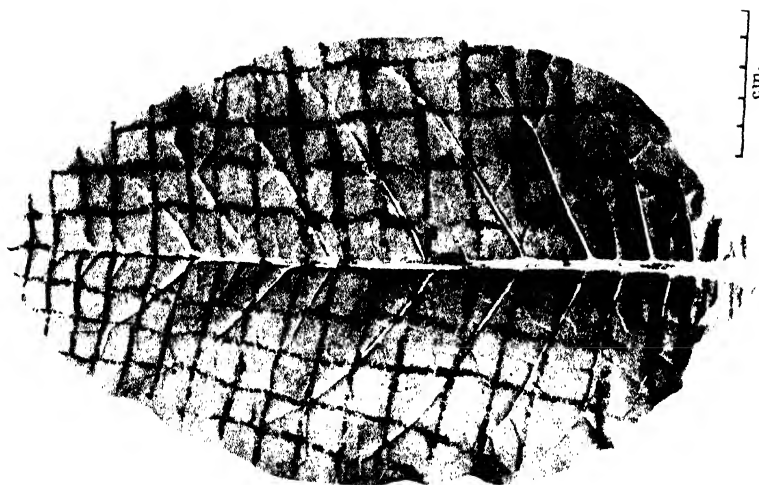


Fig. 1.

- (10) McCUBBIN, W. A. and SMITH, F. F. (1932). Rate of virus spread in tomato plants. *Science*, N.S. LXVI, 486.
- (11) SAMUEL, G. (1931). Some experiments on inoculating methods with plant viruses, and on local lesions. *Ann. App. Biol.* xviii, 494-507.
- (12) SHEFFIELD, F. M. L. (1933). The development of assimilatory tissue in solanaceous hosts infected with aucuba mosaic of tomato. *Ibid.* xx, 57-67.

EXPLANATION OF PLATE X.

Fig. 1. A leaf of tobacco which had been marked with 1 cm. squares a week previously.

Fig. 2. A wedge-shaped portion removed from the internode of a tomato plant to isolate a leaf and its axillary bud from the upper portion of the stem.

(Received August 8th, 1933.)

[FROM THE ANNALS OF APPLIED BIOLOGY, VOL. XXI, No. 2,
pp. 206-224, MAY, 1934.]
[*All rights reserved.*]

THE PHYSIOLOGY OF VIRUS DISEASES IN PLANTS

VI. SOME EFFECTS OF MOSAIC ON THE METABOLISM OF THE TOMATO

By JOHN CALDWELL.

*(Department of Plant Pathology, Rothamsted Experimental
Station, Harpenden, Herts.)*

(With Plates XI and XII and 7 Text-figures.)

CONTENTS.

	PAGE
Introduction	206
Previous work	207
Materials and methods	209
The effect of the disease on the appearance of the host plant	210
The stage of development of virus-diseased plants	212
The effect of the disease on the dry matter content of the tissues	212
The effect of the disease on the carbohydrate content of the tissues	213
The effect of the disease on the nitrogen content of the tissues	214
The effect of virus disease on the respiratory mechanism	214
Methods and materials	215
The respiration of plants inoculated as seedlings	216
The respiration of plants inoculated at later stages	218
The output of CO ₂ in nitrogen	219
The output of CO ₂ in oxygen	221
The output of CO ₂ in terms of the D.M.C.	221
The output of CO ₂ in terms of the nitrogen content	222
Summary	222
References	223

INTRODUCTION.

ALTHOUGH the effect of virus diseases on the metabolism of the host plant must obviously be considerable, the physiological study of the diseased plants has, generally, been neglected in the past in favour of the examination of their cytology and symptomatology. Various workers have, from time to time, reported on the alteration of the metabolism of

plants as a consequence of virus diseases, but the analyses which have been made have been largely concerned with the chemical aspects, and little work has been carried out on the more strictly physiological side.

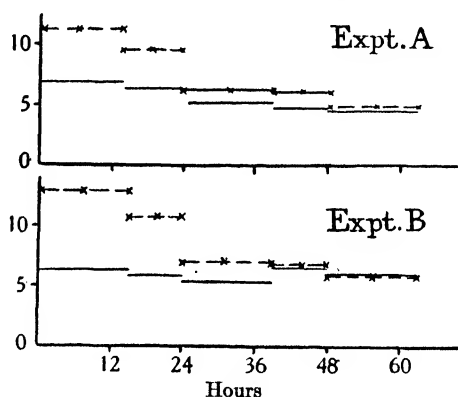
The two main processes which would most likely be affected by virus diseases are photosynthesis and respiration. That virus diseases have a definite effect in many instances on the growth of the host is evident, but the analysis of growth is difficult and not sufficiently worked out to make available much of value from its study in this connection.

Photosynthesis, which so often has been used in the past as an index of metabolism, is in these diseases of less interest than respiration because of the obvious reduction in chlorophyll content which is usually characteristic of the disease. The reduction of the amount of chlorophyll would necessitate a complicated examination of the efficiency of the photosynthetic activity if this were to be used as a criterion of metabolism. In respiration, however, there is a system, the action of which is continuous, and on which external conditions exert an influence which is fairly clearly comprehended.

PREVIOUS WORK.

There are in the literature various papers dealing with the chemical metabolism of virus-diseased plants. Woods(17) found that the oxidase content of diseased tissue was higher than that of normal. Some of his conclusions, based on the results of his work, have been challenged by Allard (1), and it is probable that he has, as Freiberg (11) suggests, mistaken the effect for the cause. Bunzel(3) found that the curly-top disease in sugar beet had the effect of reducing the sugar content of the leaves, while the nitrogen content remained unchanged. In the roots, however, the total nitrogen was increased and the sugar content decreased. Jodidi(12, 13) and others worked on the alteration in the nitrogen content of spinach and cabbage caused by virus disease and found a decrease of nitrogen in the diseased plants. True *et al.*(15) found an accumulation of carbohydrate in diseased spinach. Freiberg(11) found differences in the carbohydrate content in the light and dark green areas in tobacco with mosaic. Campbell(5) found that the leaf-roll disease of potato induced the accumulation of carbohydrates while it had no effect on the nitrogen content. Many workers have dealt with the interesting problem of the accumulation of starch in the leaves of potatoes with leaf roll and that of the translocation of the sugars through the necrosed stems.

Few workers have attempted to assess the effect of virus disease on the fundamental metabolism of the diseased plants. Thung⁽¹⁴⁾, working at Wageningen, studied the effect of leaf roll on various aspects of the metabolism of the potato. One of the aspects on which he touched was the respiration as measured by the CO_2 output. He found that the CO_2 output of the diseased tissue was higher per gram dry weight or wet weight per hour than was that of the normal. Taking into consideration the increased starch content of the diseased leaves, he concludes that the enzymatic processes are not influenced by the virus. An examination of the data which Thung furnishes on the liberation of CO_2 leads one to the view that his results are very inconclusive. The two curves in Text-fig. 1 of the present paper show the results of the two experiments which most



Text-figure 1. Graphs showing the CO_2 output of detached leaves of healthy (solid line) and leaf-roll potato plants (broken line). (From Thung.)

nearly correspond to those described in the later portion of this paper, the data being plotted as is done later for that of the tomato experiments. It can be seen that the amount of CO_2 produced by the diseased tissue is not consistently more than that produced by the healthy, and that it would, in these experiments, be quite competent to argue that, had the experiment continued without injection of the leaves by water, the tendency would have been for the output of CO_2 of both sets of leaves to have reached similar values. From the values obtained it is questionably desirable to attempt to deduce any conclusions. As can be seen from the figure, almost any hypothesis might be advanced with more or less conviction and with equal justification.

Dunlap (6, 7) has more recently in a series of papers discussed the carbohydrate content of virus-diseased plants. He has found that as

regards the reaction of the host, two main groups of virus diseases of the chlorotic type appear to exist. In the mosaics proper the effect of the disease is to increase the total nitrogen content and to decrease the carbohydrate content of the affected plants. In the yellows diseases, on the other hand, the converse holds, *i.e.* the nitrogen is decreased and the carbohydrate increased. The C/N ratio of mosaic leaves was therefore reduced and that of yellows increased. Respiration was found to be increased in young tissues affected with virus diseases and to be decreased in older tissues, as compared with the healthy controls. The method Dunlap used to determine the CO₂ output is not above suspicion, and it would appear that, to some extent, he was measuring not respiration in air alone but the CO₂ output of leaves in an atmosphere of less than normal oxygen content.

Brewer *et al.* (2), working on the effect of mosaic of tomato, found that a reduced carbohydrate content and a normal nitrogen content was characteristic, this giving a lower C/N ratio than the healthy. Woods has suggested that tomato mosaic causes an accumulation of starch in the leaves.

Elmer (9) has shown that the effect of mosaic disease in tomato is greatly to alter the composition of the chlorophyll, and Eckerson (8) has demonstrated cytologically the disintegration of the chloroplasts in tomato mosaic, though her observations mainly concerned themselves with a motile organism, the existence of which as the virus agent has not been established.

MATERIALS AND METHODS.

For the experiments here recorded the virus disease aucuba or yellow mosaic of tomato was studied in the host *Lycopersicum esculentum* var. Kondine Red, or in tobacco *Nicotiana Tabacum* var. White Burley. Where this arrangement has been departed from specific mention is made: otherwise the procedure was the same in all cases. Leaf tissue of infected plants of tomato was macerated with water in a mortar in the proportion of 1 gm. of tissue, other than petioles and midrib, to 2 c.c. of distilled water. This material was allowed to stand on one side, usually overnight, in the laboratory. It was then filtered through muslin and all the larger masses of tissue removed. Thereafter it was passed through fluted filter paper impregnated with fuller's earth. This had the effect of removing all the suspended chloroplast material and cell débris and did not apparently reduce the virulence of the agent. The resulting material was dark brown in colour and quite clear. This was used as the source of

inoculum. Inoculations were made by rubbing the leaves of experimental plants with a piece of cotton-wool soaked in this liquid. The rubbing was done in such a way as to break as many hairs as possible without extensively damaging the mesophyll cells of the leaves. As a consequence of this treatment no local lesions appeared on the rubbed leaves in the tomato, but systemic infections appeared after an appropriate incubation period.

THE EFFECT OF THE DISEASE ON THE APPEARANCE OF THE HOST PLANT.

The most evident symptom and the one which has characterised the disease is the appearance on the tomato plant of yellow areas. These develop four or five days after inoculation in summer time and after a rather longer period in dull weather. As the leaves develop, the chlorotic areas do not increase in size so rapidly as the green tissues, and the adult leaves are crinkled and distorted. The affected leaves are always smaller in size than the normal, and in summer when the growth of the controls is very rapid the difference in size is very pronounced. It is abundantly clear from macroscopical observation that the effect of this virus on the tomato plant is to inhibit the formation of chloroplasts to a greater or less extent. It has been regularly noticed that in our cultures some plants were much more affected by the disease than others. This led to a detailed examination of the effect of the age of the plant on the response to infection. Mention has been made of the fact that the intensity of symptoms is dependent on the light conditions, and the general conclusion may be drawn that under bright light conditions, especially in summer, the symptoms are much more clearly marked and the chlorosis is more pronounced. At the same time, under similar conditions it has been found that cultures of plants inoculated as seedlings show very much more extensive distortion and chlorosis than those inoculated at a later stage of development. The reason appears to be, as has been pointed out in earlier papers of this series(4), that the virus induces symptoms only in those leaves which develop subsequently to inoculation, and that all leaves which are fully developed at the time of inoculation do not develop symptoms. The leaves which were developing at the time of infection are in an intermediate condition, and the symptoms occurring on them are variable. In any plant, therefore, which is infected at a fairly late stage of development there are three regions. In the upper part the leaves have all well-developed symptoms of mosaic, in the middle part the symptoms are less evenly distributed, often only a few yellow flecks being present, and in the lower part the leaves are normal in

appearance. The non-appearance of symptoms is not indicative of absence of the virus agent from the tissues, and the virus can be extracted from all parts of an affected tomato plant after an appropriate interval of time.

The fact that the virus has no effect on the preformed chloroplasts and that the proportion of the plant deformed by the agent depends on the stage of development at infection has important effects on the metabolism of the host. The amount of reduction in the photosynthetic activity of the plant clearly depends at least in part on the reduction in the chlorophyll content. Since, therefore, the number of chloroplasts depends more or less on the leaf area of the plant, those plants which have been inoculated as seedlings will have the most reduced photosynthesis. The consequence is that the plants which have been inoculated as seedlings have no normal leaves whatsoever, and as a result of the initial reduction of chlorophyll content are much more markedly starved as compared with the controls than are those which, having been inoculated later, have a number of leaves on the lower part of the plant which by normal photosynthesis generally increase the size of the plants. Plate XI is a photograph of three plants all of the same age. At the time the photograph was taken they were three months old. That on the extreme left is the uninoculated control. The middle plant was inoculated with aucuba mosaic when it was in the fifth normal leaf condition, and that on the right was inoculated as soon as the foliage leaves were old enough to be inoculated with the virus. It can be seen that the lower leaves of the middle plant are normal in appearance and size, and that the symptoms are confined in the main to the upper leaves which are very much more reduced in size and are distorted and chlorotic. The leaves of the plant on the right are all very much reduced in size and are distorted and chlorotic. Clearly the fact that the middle plant is not so reduced in size as is the right-hand one is attributable to the fact that the lower leaves on the former were able to carry on normal photosynthesis and to produce carbohydrates which to some extent supplemented the reduced supply from the chlorotic leaves at the tip. The leaves of the right-hand plant having been reduced and chlorotic from the beginning had no normal supply of carbohydrates on which to draw and are therefore always reduced in size and the plant starved. This consideration is of importance in the study of the respiration of the healthy and diseased tissue and is more fully dealt with in a subsequent section.

THE STAGE OF DEVELOPMENT OF VIRUS-DISEASED PLANTS.

Despite the fact that the size of the plant is much affected by the disease and the metabolism is completely disturbed, the stage of development is unaffected by the virus. A study of the three plants in Plate XI, reveals the fact that despite the considerable disparity in size each of the plants is at the same stage of development. In each case the flowers of the first truss are just on the point of opening. There are the same number of leaves on each of the three although the leaves on the plant infected as a seedling are much smaller in size than those of the other two plants, and the upper leaves of the plant infected later are reduced in size compared with those of the control. This point is even more clearly seen in the two plants in Plate XII. The plant on the left had been inoculated with aucuba mosaic as a seedling, that on the right is the control of the same age. The appearance of the plants is very different, yet one is merely a reduced replica of the other. They have the same number of leaves, and the first flower truss on each can be seen projecting from the stem just above the branch which has developed in each from similar axillary buds.

THE EFFECT OF THE DISEASE ON THE DRY-MATTER
CONTENT OF THE TISSUES.

The effect of the disease on the appearance of the plant has been discussed, and this section is devoted to a consideration of the effect of the virus on the dry-matter content of the plant. Some typical results are detailed below. The leaves of the two plants shown in Plate XII, were removed and weighed. The weight of the leaves was 88.0 gm. and 20.1 gm. for the control plant and the diseased respectively. The leaf tissues therefore of the diseased plant were very much smaller in amount than that of the control. This disparity was further borne out in the dry-matter contents. This was obtained by drying the leaves at 90° C. until such times as consecutive weighings varied by only 0.01 gm. The percentage of dry matter in the control was 12.9 per cent. and in the diseased plant was 7.8 per cent. Both plants had been grown under comparable conditions, and both plants were kept equally watered so that no temporary disturbance in turgidity should occur.

This difference is of the order expected in these experiments, and the general conclusion to be drawn from a considerable number of observations is that, in plants on which the inoculations have been made at an early stage and of which the whole period of development has been

characterised by the reduction of chlorophyll and the subsequent general starvation of the plant, the dry-matter content is of the order of 7-8 per cent. In plants in which inoculations took place at a later date and which, therefore, before infection had developed normal photosynthesis, the dry-matter content was of the order of 10-12 per cent. In the healthy controls kept under similar conditions and grown in soil as nearly as possible equally moist the dry-matter content of the leaves was of the order of 14-15 per cent. The reduction in dry-matter content, expressed as a percentage, is therefore in the case of seedling inoculated plants as high as 50 per cent. and is very high in the chlorotic leaves of plants infected at a later stage in their development—about 30 per cent.

The same differences are to be found in the tops. The upper parts of the plants were removed, weighed and dried as were the leaves, and distinction could be drawn between the three groups in the same way as between the leaves of the plants. This difference is of interest in connection with the data on respiration.

THE EFFECT OF THE DISEASE ON THE CARBOHYDRATE CONTENT OF THE TISSUES.

The effect of the virus of yellow mosaic is to decrease the chlorophyll content of the tissues. As a consequence, the carbohydrate content will probably also be reduced. A complete survey of the alteration in the carbohydrates formed has not yet been made, but numerous estimations have been made of the total carbohydrate content of healthy and diseased tomato tissue. The analyses have been carried out by me and by two of my colleagues, Drs Norman and Jenkins, of the Department of Fermentation. Actually three methods were used, viz. a modified Fehling's method, the Hagedorn-Jensen method, and the Schaffer-Hartman method. The samples were collected usually about midday and were extracted for 1-2 hours with boiling 1 per cent. HCl in a sand bath. Thereafter the tissue was filtered off through muslin and washed with boiling water. After the filtrate had cooled it was made neutral to phenolphthalein with NaOH. The protein was precipitated with basic lead acetate and the excess lead removed with di-sodium hydrogen phosphate. The volume was then made up to 250 c.c. and samples taken for analysis.

As would be expected, the different samples varied very much from time to time, but there was consistently more carbohydrate in the normal than in the diseased tissue taken at the same time. The tops of plants inoculated as seedlings always contained least total carbohydrate, the tops of normal plants most, and the tops of plants inoculated after

four or more foliage leaves had formed was intermediate in position. Table I shows the kind of differences found.

Table I.

The total carbohydrate content of tops of tomato plants.

Normal plant	Plant inoculated at 5th leaf stage	Plant inoculated as seedling
1.6 %	1.3 %	1.1 %

THE EFFECT OF THE DISEASE ON THE NITROGEN CONTENT OF THE TISSUES.

The effect of the virus on the nitrogen content of the plants is less marked than on the carbohydrate content. The total nitrogen was estimated by the Kjeldahl method. The results were by no means consistent—sometimes the healthy tissue had a slightly higher nitrogen content than the virus tissue and sometimes the reverse held. When the virus tissue contained the larger amount of nitrogen, the difference could usually be attributed to the lower carbohydrate content, and it appeared that the effect of the disease was not to affect the nitrogen content of the plants.

THE EFFECT OF VIRUS DISEASE ON THE RESPIRATORY MECHANISM.

Reference has already been made to the work of Thung and Dunlap on the respiration of tissues affected with virus disease. Thung worked on the diseased leaves of potatoes with leaf-roll, as also did Esmarch (10). Thung found that the CO₂ output of virus leaves was slightly higher than that of normal, and concluded, from this observation, that, since the starch content of the leaves is much increased in this disease and the respiration is not disproportionately increased, the enzyme system must be of the same order in both healthy and diseased. In other words, that the enzymes were not greatly affected by the disease. As has been noted, there are some criticisms of Thung's methods which suggest themselves, especially as regards the validity of the deductions from the rather tenuous evidence he has available.

Whitehead (16) has reported, shortly, on the effect of leaf-roll on the respiration of potatoes, and has shown that the effect varies with the stage of development of the tubers. In general, however, the disease has the effect of increasing the CO₂ output of the tubers in air. "Anaerobic respiration," he says, "is unaffected by the virus—the tuber whether diseased or not, producing 70–80 per cent. of the CO₂ evolved under aerobic conditions."

Dunlap's work has also been discussed above, and it has been suggested that the method he employed was not wholly satisfactory. Not only is it uncertain that the caustic soda would absorb the CO_2 which is liberated, but the period over which the experiment was carried out is probably too short to make the results conclusive. At the same time, short as the period was it was probably long enough to induce anaerobic respiration, so that various unconsidered factors may have entered into the experiment.

Dunlap's general conclusions are as follows: "mature diseased leaves produce less CO_2 than similar healthy leaves, in the case of both the mosaic and yellow types of disease—the respiration rates of young leaves were found to be somewhat increased by the diseased condition—there seems to be no consistent evidence that variations in the respiratory rate due to the diseased conditions are brought about by variations in the carbohydrates or nitrogen controls of the leaves examined." In general, the findings of the various workers who have measured the respiration of diseased tissues have been conflicting, and no very clear-cut data have been available.

METHODS AND MATERIALS.

In the experiments which are outlined below, the material used in the respiration chamber was healthy and diseased tissue of the tomato (*Lycopersicum esculentum*) variety Kondine Red. These plants were grown in the glasshouses here, and the diseased plants had been infected with aucuba or yellow mosaic of the tomato, either as seedlings or at a later stage. The inoculation was made by rubbing a few of the lower leaves with a cotton-wool pad, soaked in macerated tissue from diseased plants. The symptoms of this disease are very well marked in summer and consist of a bright yellow mosaic with some leaf distortion. In the later experiments, either leaves or the whole top of plants were used, and certain precautions were taken to note the time of infection of the host plant. The reason for this is obvious from the data set out below. A known weight of leaves, or of apical shoot and leaves ("top"), was put into a glass container. Two containers were used, one for the diseased material, the other for the healthy, and they were put together into a water bath which was kept at a temperature of 25°C . A stream of CO_2 -free air, brought from outside the laboratory to avoid contamination with fumes, was led through each of the chambers. Each stream was then carried through a series of Pettenkofer tubes so arranged in the apparatus devised by Blackman that three hourly samples of the CO_2

liberated were collected in baryta in each of the tubes. The CO_2 was collected in $M/15 \text{ Ba(OH)}_2$ which was back-titrated with HCl to measure the amount of free alkali. A simple calculation gave the CO_2 equivalent to the acid required to neutralise the free baryta—phenolphthalein being used as the indicator.

In the first experiments, the material, either leaves or tops, was chosen from virus plants, without regard to the time of inoculation. It was assumed that diseased material was material which was showing symptoms of aucuba mosaic. It soon became evident, however, that the diseased tops fell into two distinct classes as regards their CO_2 output, and it was found that the distinction was associated with the time of inoculation.

This association is not surprising in the light of the results outlined above. It has been shown previously that the amount of symptoms shown by the inoculated plants depends on the amount of growth following inoculation. The more growth the plant has made before inoculation, the greater will be the supply of carbohydrate available to the diseased plant. The stages of development of the inoculated plant must, therefore, be taken into consideration when selecting material for respiration work.

When the data for the CO_2 output of healthy and virus tops are plotted graphically, as has been noted, there are two types of curves. In the first type the CO_2 output of the healthy leaves is higher initially than that of the diseased. In all cases the CO_2 output is expressed in mg. per three-hour period per 10 gm. initial fresh weight. If it be taken on the dry weight (per gm. dry weight) the difference between the virus and healthy tissues is even more pronounced, since the dry-matter content of the healthy plants is much higher than that of the diseased.

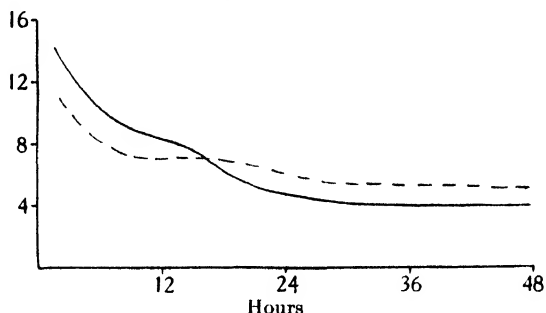
THE RESPIRATION OF PLANTS INOCULATED AS SEEDLINGS.

Initially the CO_2 output of the healthy tops is of the order of 14 mg. of CO_2 per 30 gm. hours, while the CO_2 output of the virus tops is 11 mg. These are representative figures from a large group of experiments. The data are shown graphically in Text-figs. 2 and 3. It may be seen from the figures that the two curves fall steadily at first, though that of the "healthy" CO_2 output falls with a slightly steeper gradient and, consequently, the curves cross at, approximately, the eighteenth hour of the experiment. Thereafter, though both curves continue to fall slowly the "virus" curve remains higher than the healthy. This means that the

CO₂ output of the virus tissue is lower at the early stages of starvation, while at the later stages it is higher than that of the normal tissue.

The advantage of taking three-hourly samples in this experiment and of not running the stream through each tube for twenty-four hours—as has been done in some work on virus respiration—is obvious. Had twenty-four-hour samples been taken the results obtained would have been that the CO₂ output of the healthy was much higher than that of the diseased, despite the fact that at the end of the experimental period the CO₂ output of the virus tissue was actually greater than that of the healthy. It is therefore desirable to take short-period samples continuously for some reasonably long period.

As may be seen from Text-fig. 2, the CO₂ output of both sets of tops tends to fall, that of the healthy at a slightly greater rate than that of the



Text-figure 2. CO₂ output in mg. per 10 gm. fresh weight.

diseased. As a consequence, when the fall in the CO₂ output becomes more gradual, and an “adjusted” state has been reached, the CO₂ output of the diseased tissue is about 6 mg. per 30 gm. hours, while the CO₂ output of the healthy is about 5 mg. per 30 gm. hours. The value of the N.R./D.R. ratio therefore, varies, (N.R. is the CO₂ output of the healthy tissue and D.R. that of the diseased). The value actually was 15/12, later it became 7/7, and latterly 5/6. It is clear, therefore, that some alteration is taking place in the reactions of the two tissues to starvation.

An explanation suggests itself if one considers respiration as being controlled by the separate mechanisms. This is the basis of the most recent hypothesis on respiration (cf. Neuberg, Blackman, etc.). The first group of enzymes is responsible for the preparation of the substrate, and the second group of enzymes for the breakdown of the substrate to CO₂, which is measured, and the other products of respiration. It has been shown by Blackman, that the effect of these groups of enzymes can be

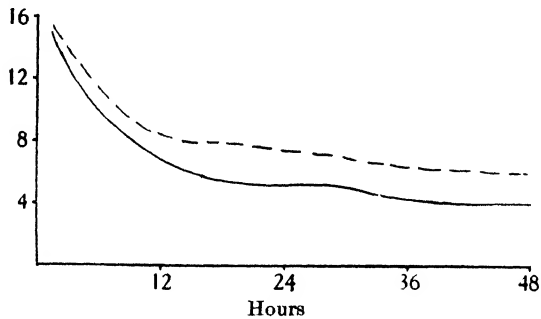
measured to some extent, and that the two groups are independent, at least, as far as some of their activities are concerned.

If the curves of CO_2 output in this case are examined in the light of the present views on respiration, one or two points emerge. The carbohydrate content of the virus tissue is much lower than that of the healthy. This has been shown by analysis. It is, therefore, quite reasonable to assume that the amount of carbohydrate available immediately to the enzymes of respiration proper, was less in virus than in the healthy tissue. The consequence of this would be that there would be a greater output of CO_2 initially from the healthy tissue. This has been found to be so in the experiments, and the facts are in agreement with the hypothesis. The crossing-over in the curves presents more difficulty, and it is suggested that the greater output of CO_2 from the virus tissue in the later stages of starvation respiration is due to the fact that while the enzymes of respiration proper (*i.e.* those responsible for the breakdown of the substrate with CO_2 , etc.) are unaffected by the disease, the enzymes which "activate" the substrate are either rendered more active, or are actually increased in amount in diseased plants. If this were correct, the amount of the less labile carbohydrates of reserve, which were available as a substrate for the respiratory enzymes would be greater in the tissue with the more efficient system—if the amounts in both instances of reserve material were sufficient not to constitute a limiting factor. The hypothesis, nevertheless, fits the facts which are available at present.

THE RESPIRATION OF PLANTS INOCULATED AT LATER STAGES.

A considerable amount of support is given by the results of another set of experiments on the CO_2 output of healthy and diseased tissues in air. The amount of chlorosis induced by the virus has been shown to depend on the amount of growth subsequent to inoculation. The tops which have just been examined were tops of plants inoculated as seedlings and, therefore, the maximum amount of reduction of chlorophyll had taken place in them. In those tissues, it has been suggested, the reduction in the amount of labile carbohydrates was responsible for the initial low output of CO_2 . If, therefore, it were possible to increase this low initial supply of labile carbohydrate, and to have the same stimulation of the enzyme system as appears in diseased tissues, it should be possible to get a larger output of CO_2 from diseased plants initially. The carbohydrate content of diseased tissues, can, as may be gathered from the foregoing, easily be increased by inoculating plants which have developed

a few normal leaves. In them the tops will develop symptoms, but a greater supply of carbohydrate will be available for the growing tissues. We have here a tissue which, if our hypothesis is correct, should have an enzyme system of increased efficiency, and a carbohydrate supply almost equal to the healthy plant. It has been shown that the carbohydrate content of the tops of plants inoculated at the fifth or sixth leaf stage, is higher than that of those inoculated as seedlings, and only just lower than that of healthy controls. When such tops are put into the respiration chambers the output of CO_2 is as shown by the curves in Text-fig. 3. The output of the healthy tissue is initially 14 mg., and it falls gradually to 4-5 mg. The output of the diseased tissue is initially 16 mg. and it falls as does the healthy until it is steady at 6-7 mg. The healthy is, therefore, similar to that of the previous experiments, while the output



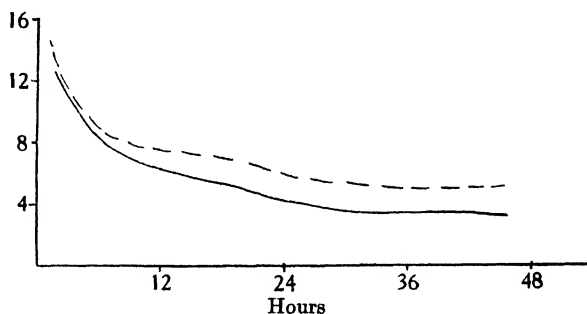
Text-fig. 3. CO_2 output in mg. per 10 gm. fresh weight.

of the virus tissue is consistently higher. The values of the ratio N.R./D.R. at the corresponding times are 14/16, 6/8 and 4.5/6.5. In no case do they reach unity. The value of the N.R./D.R. ratio at the adjusted phase was 5/6 in the first set of experiments and now N.R./D.R. = 5/7, there was, therefore, an excess of output of CO_2 by the tissues when the enzyme efficiency alone was increased by 20 per cent. and by another 20 per cent. when the carbohydrate was in greater amount.

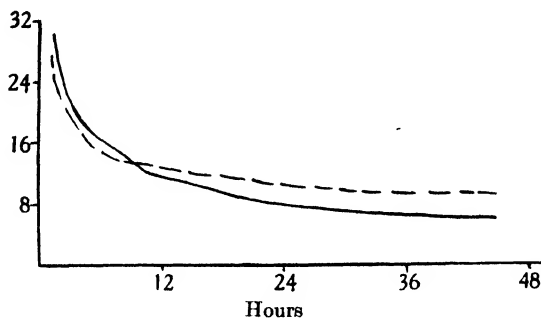
THE OUTPUT OF CO_2 IN NITROGEN.

If the view be justified that the effect of aucuba disease on the tomato plant is to increase the efficiency of that part of the enzyme system which is responsible for "activation" or "glycolysis" or some other part of the reaction which takes place before the breakdown of the substrate to CO_2 , etc., then it follows that the breakdown of substrate anaerobically should be increased by the disease. With comparable

amounts of carbohydrate, the enzymes of the virus plants should be able to break down the substrate in greater amount than do the enzymes of the healthy controls. This point was examined by estimating the CO_2 output of the tops of healthy plants and of plants which had been inoculated with aucuba at a late stage in their development. The respiring tissues were kept in an atmosphere of nitrogen. As has been seen, the carbohydrate content is but little reduced by late inoculation.



Text-fig. 4. CO_2 output (in nitrogen) in mg. per 10 gm. fresh weight.



Text-fig. 5. CO_2 output (in oxygen) in mg. per 10 gm. fresh weight.

In Text-fig. 4 there are two curves—one representing the CO_2 output of healthy tops kept in the dark in a stream of nitrogen at a constant temperature of 25°C ., the other the CO_2 output of virus tissue under similar circumstances. It may be seen that the output in both cases is of the same order initially, but that as soon as the value of the CO_2 output falls off the virus tissues produce more than the healthy. Since the carbohydrate content of both tissues is comparable, and since the absence of oxygen precludes oxidation proper, it would appear that the difference is to be attributed to the more extensive breakdown of the

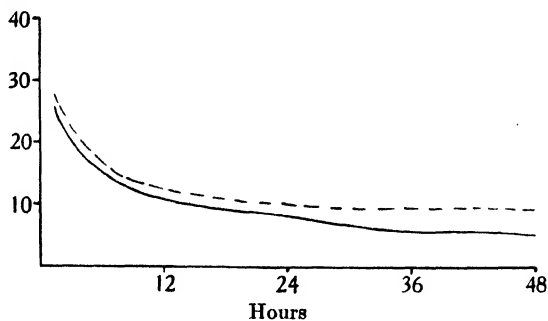
carbohydrate reserves by the more efficient enzyme system. This bears out the hypothesis which was advanced before.

THE OUTPUT OF CO_2 IN OXYGEN.

The output of CO_2 in oxygen is higher from the virus tissue than from the healthy, when tops are taken from healthy plants and from plants inoculated after half-a-dozen leaves had developed. In Text-fig. 5, the curves represent the CO_2 output from the two sets of tissues. Here again the virus tissue has the higher output.

THE OUTPUT OF CO_2 IN TERMS OF THE D.M.C.

It may be argued that the higher CO_2 output from the virus tissues is due, not to the unusual activity of these tissues but to the alteration in the water content or to the alteration in the C/N ratio. In an examina-



Text-fig. 6. CO_2 output in mg. per gm. dry matter.

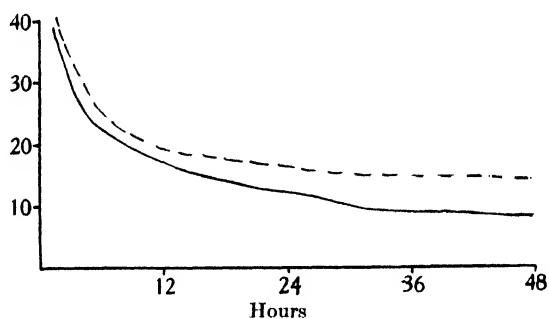
tion of this point the values of the CO_2 output are recalculated, not in terms of the initial fresh weight but in terms of the dry matter and of the nitrogen content. The expression of the CO_2 output in terms of dry weight and of nitrogen overcomes any objection which may arise by reason of the greater water content of the healthy material, should the conditions of growth of the healthy and of the virus plants be different.

In Text-fig. 6 is shown graphically the output of CO_2 from healthy and from virus tissues, expressed in terms of the residual dry matter. This necessitated the drying of the tops after experimentation in an electric oven at 85°C . until two consecutive weights did not vary by more than a milligram. The weight therefore is the dry weight after the respiration measurements are complete—the residual dry matter. The amount of carbohydrate used up in respiration over the period of experiment is slight, however, and no correction has been made for it,

since the amounts would be the same, approximately, in both the healthy and the diseased tissue. The graphs of the CO_2 output show that the virus output is again greater than the healthy taken on this basis.

THE OUTPUT OF CO_2 IN TERMS OF THE NITROGEN CONTENT.

The expression of the CO_2 output in terms of residual dry matter obviates any difficulty which might arise from a higher water content of the healthy or virus tissues, but one other consideration may arise. The unusual CO_2 output of the virus tissue might be accounted for by the fact that the protein-carbohydrate content of that tissue had been altered by the disease. It might be argued that the slightly increased nitrogen content of the virus tissues would have the effect of making available a larger amount of protein per unit of carbohydrate and that this made for greater apparent efficiency. Text-fig. 7 illustrates graphically



Text-fig. 7. CO_2 output in mg. per 800 mg. nitrogen.

the CO_2 output of the healthy and of the virus tissues when the CO_2 output is expressed in mg. of CO_2 per 100 mg. of nitrogen in the tissue. The nitrogen content was assessed by the Kjeldahl method on the top after the experiment was completed. It will be seen from these curves that the CO_2 output of the virus tissue is higher than that of the healthy per unit of nitrogen, and that the nitrogen content is not the controlling factor.

SUMMARY.

The literature dealing with the effect of virus diseases on the metabolism of the host plants is briefly summarised. Results are presented of work which has been carried out on the aucuba or yellow mosaic in tomato. The effect of time of inoculation has been studied in some detail. It has been found that the plant is generally reduced by the

disease, and that the carbohydrate and dry-matter content of the diseased plants is less than that of the controls. The stage of development of the plant is not apparently affected by the disease; the diseased plants, though reduced in size, have the same number of leaves and flower trusses as the controls. The nitrogen content is not materially affected by the disease. The effect of the disease on the respiratory mechanism of the host tissues has been examined, and it has been established that the CO_2 output of these tissues is higher than that of the controls. This is found when the output is expressed in mg. of CO_2 per three-hour period in terms of the initial fresh weight, the residual dry-matter content or of the residual nitrogen content. The higher CO_2 output is also found in respiration in oxygen or in nitrogen. This has been attributed to an increase in the efficiency of the enzyme system of the diseased plants.

This work was carried out under the auspices of the Empire Marketing Board.

REFERENCES.

- (1) ALLARD, A. A. (1916). Some properties of the virus of the mosaic disease of tobacco. *Journ. Agric. Res.* vi, 649-74.
- (2) BREWER, P. A., KENDRICK, J. B. and GARDNER, M. W. (1926). Effect of mosaic on the carbohydrate and nitrogen content of the tomato plant. *Phytopath.* xvi, 843-51.
- (3) BUNZEL, H. H. (1913). Biochemical study of the curly top of sugar beets. *U.S. Dept. Agric. Bur. Plant. Ind. Bull.* No. 277, pp. 1-28.
- (4) CALDWELL, J. (1933). The Physiology of Virus Diseases in Plants. III. Aucuba or yellow mosaic of Tomato in *Nicotiana Glutinosa* and other hosts. *Ann. App. Biol.* xix, No. 2, pp. 144-51.
- (5) CAMPBELL, E. G. (1925). Potato leaf-roll as affects the carbohydrate content and nitrogen content of the host. *Phytopath.* xv, 427-30.
- (6) DUNLAP, A. A. (1930). The total nitrogen and carbohydrates and the relative ratio of respiration in virus-affected plants. *Amer. Journ. Bot.* xvii, 348-57.
- (7) ——— (1931). Carbohydrate variations accompanying the mosaic disease of tobacco. *Ibid.* xviii, 328-36.
- (8) ECKERSON, S. H. (1926). An organism of tomato mosaic. *Bot. Gaz.* ii, 204-9.
- (9) ELMER, O. (1925). Transmissibility and pathological effects of the mosaic disease. *Journ. Agric. Exp. Stat. Res. Bull.* No. 82.
- (10) ESMARCH, F. (1932). Die Blattrollkrankheit der Kartoffel. *Monographien zum Pflanzenschutz.* Berlin.
- (11) FREIBERG, G. W. (1917). Studies in the mosaic diseases of plants. *Ann. Mo. Bot. Gard.* iv, 175-232.
- (12) JODIDI, S. L., KELLOGG, E. H. and TRUE, R. H. (1918). Nitrogen metabolism in normal and blighted spinach. *Journ. Agric. Res.* xiii, 385-408.
- (13) JODIDI, S. L., MOULTON, S. C. and MARKLEY, K. S. (1920). The mosaic disease of spinach as characterised by its nitrogen constituents. *Journ. Amer. Chem. Soc.* xlii, 1061-70.

- (14) THUNG, T. H. (1928). Physiologisch Onderzoek met Betrekking tot het Virus der Bladrolziekte van der Aardappelplant. *Tijdschrift over Plantenziekten*, xxxiv.
- (15) TRUE, R. H. *et al.* (1918). Physiological studies of normal and blighted spinach. *Journ. Agric. Res.* xv (7).
- (16) WHITEHEAD, T. (1931). Respiration of healthy and leaf-roll potatoes. *Nature*, cxxviii, 967.
- (17) WOODS, A. F. (1902). Observations on the mosaic disease of tobacco. *U.S. Dept. Agric. Bur. Plant Ind. Bull.* xviii, 1-24.

EXPLANATION OF PLATES XI AND XII.

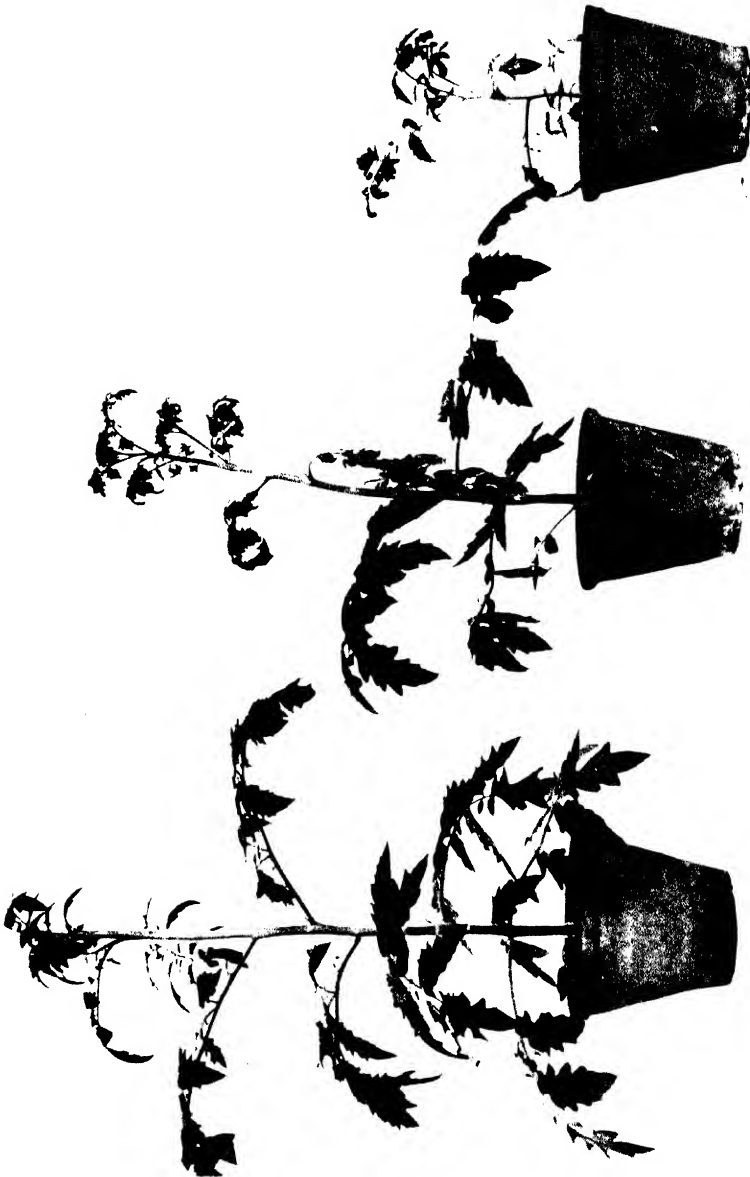
PLATE XL.

The plant on the right was inoculated with *Aucuba mosaic* as a seedling, the plant on the left is an uninoculated control and the middle plant was inoculated in the 5th leaf stage.

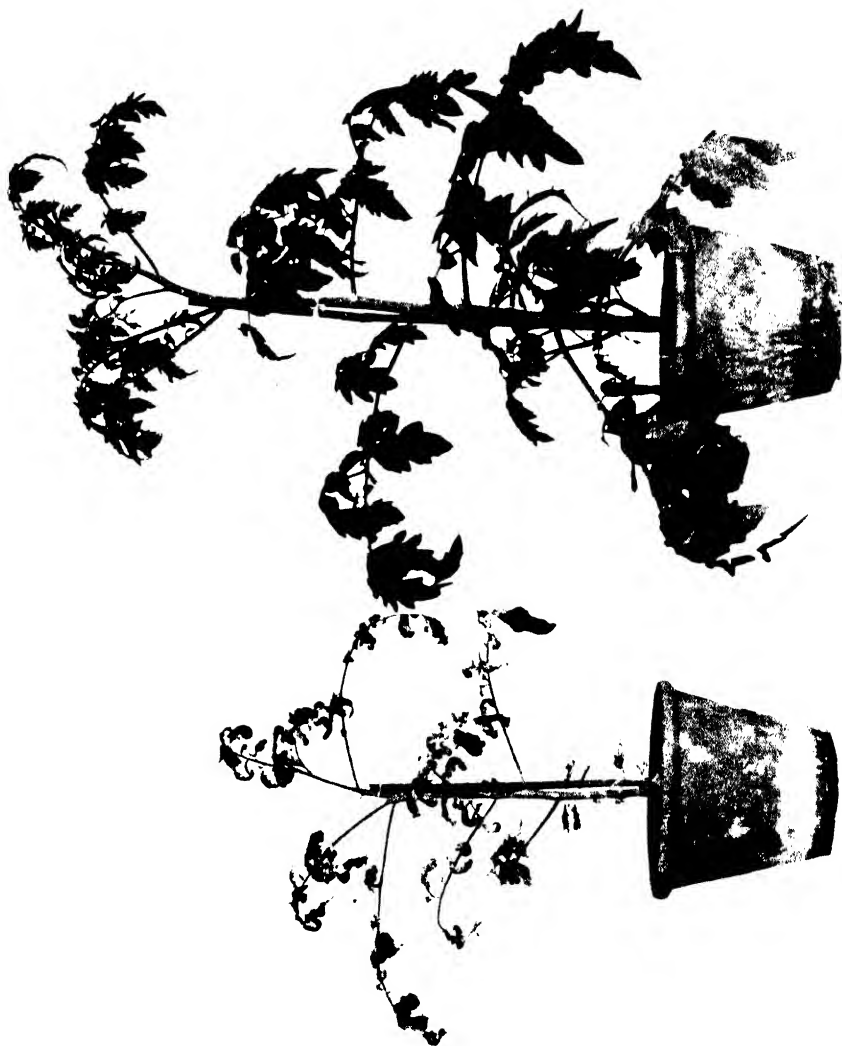
PLATE XII.

The plant on the left was inoculated as a seedling, the plant on the right is a control.
The two plants are at the same stage of development.

(Received October 4th, 1933.)



CALDWELL.—THE PHYSIOLOGY OF VIRUS DISEASES IN PLANTS (pp. 206-224).



FROM THE ANNALS OF APPLIED BIOLOGY, VOL. XXII, No. 1,
pp. 68-85, FEBRUARY, 1935.]

[All rights reserved.]

THE PHYSIOLOGY OF VIRUS DISEASES IN PLANTS

VII. EXPERIMENTS ON THE PURIFICATION OF THE VIRUS OF YELLOW MOSAIC OF TOMATO

BY JOHN CALDWELL.

*(Department of Plant Pathology, Rothamsted
Experimental Station, Harpenden.)*

(With Plates IX and X.)

CONTENTS.

	PAGE
Introduction	68
Materials and methods	70
Preliminary experiments	71
The elution of the virus from the basic lead acetate precipitate .	73
The effect of pH on the virus	73
The amount of nitrogen in the phosphate eluates	74
The separation of the virus from the protein	75
The nitrogen content of the purified crystals	78
The precipitation of the protein in the juice	79
Discussion	81
Summary	83
References	84
Explanation of Plates IX and X	85

INTRODUCTION.

THE study of the nature of the virus, its chemical composition and its structure, has been rendered more difficult by the presence of plant-products in infectious juice. Various attempts have been made, therefore, from time to time, to obtain the virus agent in a medium containing as little extraneous material as possible; only by this means will any detailed study of the agent be possible. Any protein precipitant may be used to remove most if not all of the virus from a juice but, of course, much more than the virus is removed. Two main considerations have to be kept in mind. The precipitant must not itself destroy the virus, nor must it be so toxic to the plant that inoculation subsequently might prevent the entry of the virus into the host tissues by reason of the

destruction of the cells round the place of inoculation. Alcohol has commonly been used as a precipitant as many virus agents are resistant to exposure to 60 per cent. or so of alcohol. Beijerinck(3) showed that the alcohol precipitate from juice of mosaic tobacco plants could be separated and dried at 40° C. without much loss of infectivity. Allard(1) and others have shown that active material could be separated from diseased tobacco juice by talc, and by aluminium hydroxide. Brewer and Kraybill(4) adsorbed the virus on charcoal and so obtained an active product. Vinson(16) showed that acetone at 0° C. gave a precipitate with virus juice. Acetone, at a concentration of 60 per cent., had already been shown by Allard not to be toxic to the virus. The acetone precipitate was highly infectious. Saturation with ammonium sulphate also gives a highly infectious precipitate. In the same note Vinson states that a solution of safranin-O causes a quantitative precipitation of the viruses from the juice; in later papers he considerably modifies this statement. Vinson and Petre(17) report that in electrodialysis the virus of tobacco mosaic is found at the negative pole—a highly surprising result which is hardly consistent with their other observations. They found that basic dyes, *e.g.* Bismarck brown and safranin, precipitated the virus and that the virus could be freed from the precipitate by treatment with picric acid and sodium carbonate, or with amyl alcohol. They also found that basic lead acetate caused a heavy precipitate from infectious juice and that the virus was eluted by alkaline phosphate solutions whereas acid phosphate solutions did not remove it. They concluded that it is “probable that the virus we have investigated reacted as a chemical substance.” Woods(19) and Freiberg(9) had suggested previously that the virus was enzymatic in nature. In a later paper(18) Vinson and Petre gave more details of their method of precipitation and elution of the virus, and report that norite and talc added to a virus juice apparently increase the degree of infectivity. The estimates they make of the amount of virus present depend on the infection of numbers of tobacco plants, and, in the main, the percentage of infection among the control plants inoculated with untreated juice seems surprisingly low. In a recent note Barton-Wright and McBain(2) claim that by using Vinson and Petre’s technique they have been able to increase the purity of the virus extract enormously. Their work is discussed later in this paper.

In the animal virus field, the position is similar. Numerous workers, particularly Kligler and Olitsky(10), and Pirie(14), Kreuger and Tamada(11) and others, have been able to remove the infective principle from a serum. So far no one has succeeded in removing the virus from a plant juice into

a protein-free medium. Clearly, the higher the degree of purity which is obtained, the more precise will be our knowledge of the chemical and physical properties of the virus and to this end much work has been, and will in the future have to be, applied. At the present moment, only Barton-Wright and McBain⁽²⁾ for tobacco virus and Bronfenbrenner⁽⁵⁾ for bacteriophage have suggested that the virus might be carbohydrate in nature.

This paper gives some account of experiments which have been carried out with a view to the purification of the virus and of observations made on the purified material.

MATERIALS AND METHODS.

For work on the purification of the virus it is clear that only viruses which have a fair resistance to storage *in vitro* and to chemical treatment are suitable. The virus of yellow or aucuba mosaic of tomato is particularly well suited for this type of work, and was used in most of these experiments. It was obtained from its host-tomato (*Lycopersicum esculentum* var. "Kondine Red") for the most part, but occasionally it was obtained from tobacco (*Nicotiana tabacum* var. "White Burley"). The symptoms of the diseases caused by this virus have been fully described before⁽¹⁵⁾. It induces systemic mosaic on both tobacco and tomato, and a local necrosis on the leaves of *N. glutinosa*. *N. glutinosa* was used as a test plant for the virus since it has been shown in an earlier paper of this series⁽¹⁾ that the number of lesions on the leaf was proportional to the concentration of virus in any infective juice.

In the preparation of the juice in all the experiments the same technique was adopted. This has been described in an earlier paper⁽⁶⁾ and consisted of crushing the leaves with twice their weight of water. This material was kept overnight at room temperature to allow of the breakdown of the tissues and of the settling out of inorganic salts of various kinds. Next day, the macerated material was filtered through muslin and a greenish juice obtained. The chloroplasts and cell debris were easily removed by filtration through filter paper slightly impregnated with fuller's earth. This material was in the work recorded in earlier papers of this series considered as the prepared juice and was shown to contain much active virus. In this paper material of this kind is designated the prepared juice.

It has been shown that the virus will persist in an active condition in this material for many years, and that chemical treatment has relatively little effect upon it.

At each stage in the experiments outlined below tests were made by inoculation into *N. glutinosa* leaves to ascertain the amount of active virus present after the preceding treatment.

PRELIMINARY EXPERIMENTS.

A slight modification of the methods of Vinson and Petre was made to facilitate the preparation of as large a quantity as possible of the virus agent. The procedure adopted was as follows. To the prepared juice were added 5 percent. of a solution of basic lead acetate prepared by the standard method. A heavy precipitate—creamy coloured when tobacco juice and brown when tomato juice was used—was thrown down. This precipitate which was removed on a centrifuge was found to contain all the virus. The supernatant liquid was normally quite free from virus particles, though occasionally one or two spots would appear on the inoculated *glutinosa* leaves. The absence of necrosis following inoculation was not due to the toxicity to the plant tissues of the lead in the solution since no more infection was observed when the lead was precipitated by the addition of di-sodium-hydrogen phosphate to the liquid. Neither the precipitate so formed nor the supernatant liquid was found to contain virus.

The basic lead acetate precipitate was suspended in five or six times its own volumes of $M/3 \text{ KH}_2\text{PO}_4$ solution and left for two hours, being carefully shaken up from time to time. Thereafter the mixture was centrifuged to separate the precipitate. The supernatant liquid was found to be wholly free from virus or to have only a few particles present in it. The precipitate contained large quantities of virus. The process of suspension in acid phosphate and separation was repeated with the same result. Thereafter, the precipitate—now much less bulky—was suspended in a like quantity of $M/1 \text{ K}_2\text{HPO}_4$ solution and left overnight. After 24 hours the mixture was centrifuged, when the supernatant liquid was made up with distilled water to the original volume of the prepared juice. This liquid was found to contain practically as much virus as the original juice; some virus did, however, remain in the precipitate as was shown by suspending it in water and using it as an inoculum. The treatment of this material and the results obtained in a series of experiments are shown in Table I.

It was found that a second suspension and separation in $M/3 \text{ K}_2\text{HPO}_4$ was usually sufficient to remove the virus from the precipitate, in which case the second K_2HPO_4 precipitate on suspension in water was found to contain practically no virus at all.

Table I.

The preparation of the purified virus material.

Treatment	pH	Virus content (Necrotic spots on leaves of <i>N. glutinosa</i>)
(1) 40 gm. of infected leaf—80 c.c. water macerated and left overnight, strained through muslin and filtered through fuller's earth gave 100 c.c. juice	Approx. 5.79	Many spots
(2) 100 c.c. juice—5 c.c. basic lead acetate solution supernatant	Approx. 7.5	No spots
(3) Precipitate of (2)—20 c.c. $M/3 \text{ KH}_2\text{PO}_4$ supernatant made up to 100 c.c.	Approx. 4.8	Few spots
(4) Precipitate of (3)—20 c.c. $M/3 \text{ KH}_2\text{PO}_4$ supernatant made up to 100 c.c.	Approx. 4.6	Few spots
(5) Precipitate of (4)—20 c.c. $M/1 \text{ K}_2\text{HPO}_4$ supernatant made up to 100 c.c.	Approx. 7.35	Many spots
(6) Precipitate of (5)— H_2O	Approx. 7.30	Some spots

It was also found that the preliminary precipitation with basic lead acetate suggested by Vinson and Petre was under our conditions undesirable since a concentration of basic lead acetate as low as 3 per cent., while it did not remove all the precipitable material from the prepared juice, did remove in most cases all the virus, the supernatant liquid being inactive on inoculation into *N. glutinosa*.

When a sufficient amount of a 20 per cent. normal lead acetate solution to cause complete precipitation was added to the treated juice it was found that the supernatant liquid contained no virus. This liquid had a pH of 5.5 and gave a heavy precipitate on the addition of basic lead acetate.

In all the experiments recorded here, therefore, there was no preliminary precipitation with lead acetate but the single precipitation with basic lead acetate, as described, was adopted.

The use of H_2S to remove the lead from basic lead acetate precipitate supernatant was tried but was found unsatisfactory, as shown by Vinson and Petre, since the treatment made the suspension very alkaline, and the results were very irregular.

Other protein precipitants were also tried such as ammonium sulphate, mercuric sulphate, hydrochloric acid and trichloroacetic acid, but their use was discontinued because of the difficulty of inoculation after their use. As has been pointed out above, only these treatments which neither destroy the virus nor are themselves toxic to the plant tissues are of any value in work on the precipitation of the virus.

THE ELUTION OF THE VIRUS FROM THE BASIC LEAD
ACETATE PRECIPITATE.

It is clear that the hydrogen-ion concentration is the major factor in the elution of the virus from the basic lead acetate precipitate as described above. Alkaline potassium-hydrogen phosphate removed the virus from the precipitate while acid potassium-hydrogen phosphate did not. A series of buffer solutions of mixed potassium-hydrogen phosphates were made up so that they had pH values of approximately:

4.5 (KH_2PO_4) 5.0 5.5 6.0 6.5 7.0 8.0 (K_2HPO_4).

The basic lead acetate precipitate was prepared in the usual way and then was suspended in each of the solutions in turn for one hour, being well shaken. After each suspension the mixture was centrifuged and the supernatant liquid treated for the presence of virus. It was found that the solutions of 4.5 and 5.0 pH had little effect on adsorption of the virus on the precipitate, that a little virus was washed off at pH 5.5 and that at the higher pH values the virus was washed off with ease.

THE EFFECT OF pH ON THE VIRUS.

Since the pH of the solution is so important a factor in the removal of the virus from the precipitate, the effect of the pH of this solution on the virus was examined. A large quantity of $M/10$ K_2HPO_4 eluate was prepared as described above. This eluate, as we have seen, has a pH of approximately 7.0–8.0 and is highly infectious when inoculated. The alteration of the pH of this material may throw down a precipitate but when this took place the whole of the material—precipitate and liquid alike—was used as an inoculum. The pH of the prepared liquid was altered by the addition of KOH solution to increase the alkalinity or of H_3PO_4 to increase the acidity. In this way it was possible to alter the pH over a wide range without the addition of ions other than the original potassium, hydrogen, or phosphate which were present in the first instance.

As a preliminary study, the KH_2PO_4 eluate of the basic lead acetate precipitate was tested and found on inoculation to induce the formation of one or two spots on each leaf. The pH of this eluate was 4.4 and it was rendered more alkaline by the addition of KOH —making a pH of 6.4. At this pH further inoculations on the leaves of *N. glutinosa* were made but no increase in the number of spots was observed. The pH of the KH_2PO_4 eluate was, therefore, not responsible for the non-infection of the *N. glutinosa* leaves.

The K_2HPO_4 eluate which was used for the major portion of the experiments had a $p\text{H}$ of 7.4. This was inoculated into a series of leaves and caused a large number of spots on each leaf. To this eluate was added H_3PO_4 until the $p\text{H}$ was 2.2. It was put on one side for six hours. At this $p\text{H}$ the liquid was cloudy, and was well shaken before inoculation. The effect on the leaf-tissues was severe and the leaves were rather burnt by the acid. Numerous necrotic lesions typical of the virus were, however, formed. It was clear that the exposure of the virus for six hours to a $p\text{H}$ of 2.2 had little effect on its activity. This was confirmed by the inoculation of the same material after the $p\text{H}$ had been adjusted to neutral. To another portion of the eluate was added H_3PO_4 to give a $p\text{H}$ of approximately 3.0. This liquid was again cloudy, and, after being left on one side for 6 hours, was found to be as infectious as the original control. There was no burning of leaves in this instance.

The alkalinity of the eluate was increased by the addition of KOH . After six hours, at a $p\text{H}$ of approximately 11.0 the solution was quite clear and no virus activity was demonstrable on inoculation. The leaves were not burnt in this instance. The activity was restored to a considerable extent on the return of the $p\text{H}$ of the juice to 6.4 by the addition of H_3PO_4 . A similar exposure of the material to a $p\text{H}$ of approximately 9.8 had no obvious effect on the virus activity either before or after neutralisation.

The $p\text{H}$ of yet another portion of the eluate was adjusted to 4.7—the $p\text{H}$ (approximately) of the KH_2PO_4 eluate. This had the effect of making cloudy the material which was inoculated on to the leaves in the usual manner. This treatment did not reduce the virus content of the liquid.

THE AMOUNT OF NITROGEN IN THE PHOSPHATE ELUATES.

It was clear that the liquid obtained by elution of the basic lead acetate precipitate in the different phosphate solutions contained protein and that in the K_2HPO_4 eluate, in which the virus was recovered almost quantitatively, there was considerable protein material. In addition there was a large amount of pigment, the removal of which presented some difficulties which have not yet been overcome. Estimations of the amount of nitrogen present at the different stages in the treatment were made and the results of two representative experiments are given below. In all the experiments the nitrogen content was reduced in the K_2HPO_4 eluate to 5–10 per cent. of that of the original juice.

It will be seen, from the data in Table II, that one-half of the nitrogen present in the prepared juice is removed in the basic lead acetate super-

natant and that, of the rest, only a portion is washed off the precipitate when the virus is eluted with K_2HPO_4 solution. It is probable that the nitrogen removed in the basic lead acetate supernatant represents inorganic and other forms of non-protein nitrogen so that a higher proportion of the original protein is recovered in the K_2HPO_4 eluate than would appear from the figures for nitrogen.

Table II.

The amounts of nitrogen present in the treated materials at different stages.

Material	pH		Nitrogen (total mg.)	
	Exp. A	Exp. B	Exp. A	Exp. B
Juice	—	5.41	320.0	562.9
Basic lead acetate super.	—	5.96	173.5	344.2
KH_2PO_4 eluate	—	4.5	48.5	23.2
K_2HPO_4 super. eluate	—	6.7	37.2	26.0
Basic lead acetate ppt.	—	—	35.8	—

THE SEPARATION OF THE VIRUS FROM THE PROTEIN.

The data recorded above show that the virus may easily be recovered, practically undiminished in quantity, in a liquid, and in the presence of a much reduced protein content from that of the prepared juice. The next step was the removal of the virus from the protein, if that were possible, or the demonstration of the nature of the virus by either direct or indirect means. A series of methods of protein precipitations were tried and in each case the protein and the virus tended to be found together. Numerous experimenters have found this, particularly with tobacco mosaic, which by reason of its resistance to ordinary treatment is very suitable for experimentation. The most recent work is that of Barton-Wright and McBain, to which allusion has been made. They found that the reconversion of the mixed phosphates in the viruliferous eluate of Vinson and Petre to KH_2PO_4 and the addition of two volumes of acetone to one of the acidified eluate resulted in the formation of a precipitate. This precipitate they found in their experiments to be largely protein. There was nothing new in the precipitation of the protein by acetone and they merely confirmed the findings of other workers that the protein took the virus down with it. The supernatant liquid in similar experiments done in this laboratory was always free of virus. The acetone present seems to have no serious effect on the leaves of *N. glutinosa* on inoculation.

Barton-Wright and McBain, however, went on to a separation of the precipitate into a colloidal (the protein) and a crystalline fraction. This

crystalline fraction, they allege, was typical of virus juice and was not found when healthy juice had been treated in exactly the same way as the virus material. They, therefore, by implication, make it appear that the virus is in a highly purified state in these crystals. Exception has already been taken to this position by the present writer (8), who has been unable to obtain crystals which are at once nitrogen-free and virus-containing. The methods and results are outlined below.

The K_2HPO_4 eluate was prepared in the usual way and was found to have a pH of 7.8. To this was added sufficient H_3PO_4 to give a pH of 4.7. This material was slightly cloudy. To it was added two volumes of acetone when a heavy precipitate was thrown down. This precipitate on examination was found to consist of two portions—a crystalline portion and a colloidal. The supernatant was free from virus and both the crystalline and the colloidal portions appeared to contain virus. There was comparatively little virus in the crystalline material but a great deal in the colloidal portion. The colloidal precipitate can readily be shown to be protein.

Up to this point the writer is in agreement with Barton-Wright and McBain. The constitution of the crystals is the point in which the differences occur. Briefly, they find that no crystals are formed when healthy juice is treated as was the infected, and that the crystals, though they contain no nitrogen, were found to cause infection on inoculation into tobacco. That is to say, they contained some virus—the amount of which is unknown.

The writer's experience has been as follows. The crystalline portion of the precipitate from infective juice was washed in acetone and water mixture (2 acetone to 1 of water by volume) and, so far as possible, all the colloidal material removed. Thereafter the crystals were washed once or twice with acetone, which was decanted off and then they were dried. These crystals differ slightly if prepared from tomato or tobacco tissue. Those from tomato are slightly brown in colour as against the white crystals from the tobacco tissue. It was found that these crystals in aqueous solution contained a small trace of virus—the tests for the presence of virus were made on *N. glutinosa* leaves and were, therefore, quantitative as well as qualitative. A little of this aqueous solution was tested serologically for the presence of protein and was found to give positive results. Similar crystals were also examined microanalytically and were found to contain a trace of organic material which charred with sulphuric acid and also enough nitrogen to make possible a certain demonstration of its presence. The serological tests were made possible

by the kindness of Dr J. M. Birkeland and the microanalyses were carried out by my colleague Mr F. J. Richards. When similar crystals were dissolved in water and reprecipitated it was found that the supernatant liquid contained a trace of virus and the resulting crystals contained less virus than the original crystals.

The microscopic examination of the crystals showed that in outline they resembled closely the crystals of KH_2PO_4 . For this reason, similar experiments were set up with healthy juice and with the reagents themselves in the absence of juice.

The healthy material on similar treatment produced crystals identical in appearance with those of the infectious juice. It is therefore evident that the presence of virus in a juice had no direct connection with the appearance of the crystals.

The addition of two volumes of acetone to one of an aqueous solution of KH_2PO_4 causes the precipitation of crystalline material, the amount increasing with the concentration of phosphate in the solution. An $M/15$ solution of KH_2PO_4 contains approximately 9.0 gm. of salt per litre. 50 c.c. contains therefore 0.45 gm. The addition of 100 c.c. (two volumes) of acetone to 50 c.c. of this solution results in the formation of dense white precipitate of needle-shaped crystals (not a faint opalescence as suggested by Barton-Wright and McBain). After half an hour there is a deposition of the crystals at the bottom of the container. At ordinary room temperature the yield of crystals is approximately 0.3 gm. from 50 c.c. of solution. The first reaction in this and in the earlier cases is the formation of bulky precipitate of needle-shaped crystals which disappear as the liquid is allowed to dry up, *e.g.* on a microscope slide, and are replaced by the larger rhombic crystals shown in the figures (Plates IX and X, figs. 1-3). The rhombic crystals have the same shape in the presence or absence of either healthy or infectious plant juice. It is clear, therefore, that the crystals are formed by the action of the only substances common to all three groups of materials, *viz.* the reagents.

The microscopical examination of the crystals during and after formation reveal some very important details. It was noticed that in the experiments involving plant juice, either healthy or infected, the needle-shaped crystals which are formed immediately on the addition of the acetone to the acidified eluate tend to be aggregated, quite irregularly, into groups round colloidal material. In these same experiments an examination of the final crystals showed that they had apparently been formed round masses of colloidal material. It is suggested that the effect of acetone on the eluate at a pH of 4-5 is to precipitate the protein and

the crystalline KH_2PO_4 simultaneously so that the colloidal material acts as a nucleus for crystal formation. The figures in Plate X illustrate the appearance of the crystals which are obtained from diseased juice, healthy juice and from the reagents in the absence of protein material. It is clear from them that the colloidal material which is present in the first two types of crystals and absent from those from the reagents alone make difficult the separation of the crystals from organic material after precipitation from an acetone-phosphate mixture.

The observation that the crystals of phosphate contained colloidal material led to the setting up of another group of experiments. In the earlier experiments the concentration of the phosphate used in elution was $M/10$. It was found, by experience, that more concentrated phosphate solutions had the effect of damaging the tissues of the leaves of *N. glutinosa* on inoculation and, therefore, were unsatisfactory in quantitative virus studies. For the purposes of precipitation, however, as has been pointed out, the higher the concentration of phosphate the greater the amount of the crystalline portion of the precipitate. A series of K_2HPO_4 solutions were prepared with concentrations of $M/10$, $M/5$, and $M/1$. These were used in the elution of the different basic lead acetate precipitated from both healthy and infected juice. Thereafter, they were acidified with H_3PO_4 to $p\text{H } 4.5$ —which, in effect, entails the conversion of the K_2HPO_4 to KH_2PO_4 —and two volumes of acetone added. It was found that with the $M/10$ eluate the greater portion of the precipitate was colloidal in nature with a small crystalline portion, that practically all the precipitate was crystalline in the case of $M/5$ K_2HPO_4 eluate, and that there was a large crystalline precipitate in the $M/1$ K_2HPO_4 eluate. In other words, the colloid material was used as the nucleus for crystal formation and, when the quantity of phosphate was sufficiently large, the protein material was all included in the crystals.

THE NITROGEN CONTENT OF THE PURIFIED CRYSTALS.

In the foregoing sections the question of the precipitation of the protein and the salts by acetone has been discussed. Some further details of the crystalline portion of the precipitate are now to be considered. The reaction immediately following on the addition of acetone to the phosphate eluate is the formation of a heavy precipitate of mixed colloid and crystalline nature—the proportions varying with the concentration of phosphate in the eluate. When $M/10$ phosphate is used only small amounts of crystals are obtained. For the purposes of these experiments involving chemical analysis, therefore, $M/5$ K_2HPO_4 solutions were used. After

elution and acidification two volumes of acetone were added. The precipitate was readily separated by decantation and the crystalline portion separated from the colloidal. The supernatant was put into a refrigerator at -5°C . As a consequence, after a few days a further precipitate was formed. This precipitate appeared to be wholly crystalline on examination and it was thought that this probably contained little of the colloid material. On inoculation into *N. glutinosa* the crystals were found to contain traces of virus, and microanalysis demonstrated in them the presence of organic material and of traces of nitrogen.

A series of microanalyses on the purified crystals were carried out. As a control KH_2PO_4 crystals were precipitated from an $M/1$ aqueous solution with acetone and were collected. These were found to contain no demonstrable nitrogen on analysis. Similar crystals were prepared from the phosphate eluate of precipitates from both healthy and virus juice and these were found to contain in the unpurified state between 0.1–0.2 per cent. of nitrogen. Actually, the difference in nitrogen content between the crystals from healthy materials and those from diseased tissue was not significant. Even those crystals which were obtained by keeping the supernatant in a refrigerator for some days—and crystals did form under these conditions—were found to contain slight traces of nitrogen.

THE PRECIPITATION OF THE PROTEIN IN THE JUICE.

In a series of experiments on the juice of infected plants results were obtained which are germane to this study. Juice prepared by filtration through fuller's earth was used in these experiments. To a portion of this juice was added two volumes of acetone. A heavy precipitate was obtained and this was removed on a centrifuge. The precipitate was made up to the original volume with water and was found to contain all the virus, the supernatant liquid being almost completely virus-free. *N. glutinosa* was used as the test-plant. A second portion of the juice was heated to 75°C . for 15 min. and a precipitate was formed. This precipitate was also centrifuged off and made up with water to the original volume. In this instance, both the precipitate and the supernatant were found to contain virus. A third portion of the juice was put into a U-tube the ends of which were closed with cellophane held in position by rubber bands. The tube was inverted so that each arm was under water in a separate beaker. Platinum electrodes were inserted into the beakers and a current of 100 volts D.C. was passed through the liquid. At the end of two hours a large precipitate had formed in the U-tube and the water in the beakers contained some quantity of electrolytes. The water in the

beakers was changed frequently and the current allowed to run for twelve hours. At the end of that time the precipitate was separated from the supernatant liquid, made up to the original volume with water, and both were inoculated into *N. glutinosa* leaves. The precipitate contained much virus, the supernatant induced the formation of only a few spots on inoculation.

To a fourth portion of the juice was added sufficient ammonium sulphate to give a saturated solution. A dark precipitate was thrown down which was separated from the supernatant by centrifugation and made up with water to the volume of the juice originally saturated. This material contained much virus. The supernatant was electro dialysed as above described, and no precipitate was formed nor was any virus found to be present on inoculation.

Yet another portion of the juice was saturated with magnesium sulphate, and the precipitate removed. This precipitate contained virus. The supernatant was electro dialysed, when a second precipitate was formed which, on inoculation, was also found to contain virus. The second supernatant was virus-free. The same juice was then diluted one in ten with water and various substances added to different portions. Liquid soap and various enzymes were used. The juice was kept exposed to the action of these substances for 24 hours at room temperature, after which inoculations were made. The results obtained are recorded in Table III.

Table III.

Effect of soap and enzymes on infectivity of juice.

Treatment	No. of spots	Treatment	No. of spots
Control juice	∞	0.25 % Taka diastase	3-6
1/500 Liquid soap	∞	2.5 % Pepsin	20
1/200 Liquid soap	1-4	2.5 % Trypsin	0-1
1/100 Liquid soap	0	2.5 % Papain	0
2.5 % Taka diastase	0-1	0.25 % Papain	0-1

Thereafter the juice to which the enzyme had been added was heated to 75° C. for 15 min. and further inoculations made. The results are recorded in Table IV.

Table IV.

Effect of heating enzyme-juice mixture at 75° C. for 15 min.

Treatment	No. of spots
Control	∞
2.5 % Taka diastase	0-1
2.5 % Pepsin	10-20
2.5 % Trypsin	∞
2.5 % Papain	0

When juice which had been exposed to liquid soap at a concentration of one in five was acidified with phosphoric acid after 72 hours' treatment a white precipitate was formed. The mixture was shaken up and inoculated into *N. glutinosa* when, despite the slight burning of the leaves, numerous spots were formed.

DISCUSSION.

From the results detailed above it is probable that any protein precipitant will remove the virus of yellow mosaic of tomato from infected juice. Whether the virus can be recovered from the precipitate or not depends on the action of the precipitants. The virus is active over a very wide range of pH —from approximately 2.5 to 10.5—and outside that range the virus is not necessarily destroyed by the acidity or alkalinity since, as has been pointed out, the neutralisation of juices of pH above and below the extremes of this range resulted in a return of the virus symptoms on inoculation. Whether, in these instances, the excessive acidity or alkalinity had temporarily inactivated the virus or whether the effect of the acidity or alkalinity was to prevent the entry of the agent into the plant is a point difficult to settle, but the weight of the evidence is in favour of the latter explanation. It would appear that, were the virus even in an inactive state, to gain entry into a broken cell the pH of the cell would restore the activity lost by reason of the excessive acidity or alkalinity of the medium.

It cannot be too emphatically stated that the effect of the reagents used on the actual cells of the host plant, *e.g.* *N. glutinosa*, is probably the main factor in the non-development of symptoms after many of the treatments to which the virus juice has been subjected. Many times it has been found that reagents which produced no actual necrosis of the leaf-tissues were effective in preventing the development of typical lesions.

From the experiments on precipitation, recorded in this and in other papers, it may be concluded that the virus is either protein in nature or is so closely adsorbed to the protein that, on any alteration of the physical state of the juice, the virus reacts as does the protein. This holds for all viruses of the tobacco mosaic group, at least, and for many others in other groups.

The results of the experiments with enzymes are of interest in this connection. Various workers have at different times recorded experiments with enzymes and, since the virus does react like a protein in so many ways, the interest of the reaction of proteolytic enzymes on the

virus is obvious. In a recent paper Lojkin and Vinson⁽¹²⁾ report the results of some experiments on the effect of enzymes on the virus of ordinary tobacco mosaic (Johnson's tobacco virus no. 1). It is of interest to note that they give no precise data regarding the concentration of the enzyme they used, although they report that some enzymes would inactivate "purified" virus but not virus in crude juice. It might well be that the concentration of the virus in the crude juice was such as to be greater than the enzyme present could inactivate, though this is improbable. They found that under the conditions of their experiment, emulsin, pepsin and yeast extract did not reduce the infectivity of the virus juice. They conclude further, that the "inactivation of the virus by enzymes is not due to adsorption—it seems likely that the inactivating effect of the enzyme solution is due to its hydrolytic effects." The present writer's results bear out some of the conclusions of Lojkin and Vinson. He has found repeatedly that pepsin even in such high concentrations as 2.5 per cent. is unable completely to inactivate the virus of yellow mosaic of tomato and that after 24 hours' incubation with 2.5 per cent. pepsin a large proportion of the virus is unaffected, as judged by the formation of necrotic lesions on the leaves of *N. glutinosa*. At lower concentrations of pepsin there is no evidence of any effect on the concentration of the virus. Under similar conditions, however, trypsin has the effect of apparently completely inactivating the same virus in 24 hours at room temperature. This, as has been pointed out before (Caldwell⁽⁷⁾), does not mean the destruction of the virus since heating at 75° C. for 15 min. restores the greater portion of the virus activity. Whether the effect of the trypsin is to inactivate the virus, which is reactivated on heating, or whether it is to prevent the adsorption of the virus by the cell walls is difficult to determine. 2.5 per cent. papain, on the other hand, inactivated the virus in 24 hours and no recovery was observed after treatment at 75° C. for 15 min., which is not effective in destroying the proteolytic activity of the enzyme. The difficulty of removing the enzyme, or at least inactivating it, has occasioned a good deal of work on this material. The general conclusion to be drawn from a large number of experiments seems to be that the enzyme may act on the broken tissue of the inoculated leaves and tend to prevent the entry of the virus into the tissues rather than that it destroys the virus itself. The available evidence of many experiments is summarised in Table V. In some experiments advantage was taken of the facts that iodine and HgCl_2 are supposed to act as inhibitors and that the optimum pH for papain activity is about 5.0 with just below 4 and above 9 as extremes (see Oppenheim⁽¹³⁾).

Table V.

The effect of papain on the virus.

Conc. of papain %	Treatment	pH	No. of spots
0.5	Immediate inoculation	—	0
0.5	Inoculation after 24 hours	—	0
0.5	Boiled before addition to virus	—	Many
0.5	Inoculation after treatment at 75° C. for 10 min. following 24 hours' incubation	—	1-2
1.0	Inoculation after 24 hours	—	0-1
1.0	Inoculation after treatment at 75° C. for 30 min. following 24 hours' incubation	—	Few spots
1.0	Washed off immediately after inoculation after incubation	—	0-1
1.0	Washed off immediately after inoculation without incubation	—	0
1.0	Immediate inoculation	5.3	0-4
1.0	Inoculation after 72 hours' incubation	5.3	2-4
1.0	+ KOH and exposed 24 hours	9.21	1-2
0.1	Exposed 24 hours	5.72	Many
0.1	" "	4.24	"
0.2	" "	3.14	"
0.2	" "	9.23	Some spots
0.2	" "	5.58	Many

From these experimental results it appears that a high concentration of papain is necessary to destroy or prevent the activity of this virus. Low concentrations are ineffective in preventing the formation of necrotic lesions on inoculated leaves of *N. glutinosa*. It has not yet been clearly established that the effect of the papain is on the actual virus and not on the tissues of the inoculated leaves, and work on this aspect of the problem is being continued.

SUMMARY.

Experiments on the purification of the virus of yellow mosaic of tomato are described and discussed. Vinson and Petre's methods of purification of the virus from infectious juice and subsequent elution with phosphate solution were slightly modified in these experiments. It was found that there was no evidence that the virus could be recovered in a crystalline form and that viruliferous material always contained traces of organic nitrogen. This virus was found to be active over a wide range of pH, viz. from 2.0 to 10.5. At the extremes of the scale, the excessive acidity or alkalinity was toxic to the inoculated leaves and adjustments had to be made before inoculation. Different protein precipitants were used in an attempt to free the virus from the proteins, and electrolytic

methods were also tried. Proteolytic enzymes were employed on the purified virus juice but the results were rather unsatisfactory. Difficulty was experienced in ensuring that the effect of some reagents was on the virus and not on the tissues of the test plants.

REFERENCES.

- (1) ALLARD, H. A. (1918). Effects of various salts, acids, germicides, etc., upon the infectivity of the virus causing the mosaic disease of tobacco. *J. agric. Res.* XIII, 619-37.
- (2) BARTON-WRIGHT, E. C. and MCBAIN, A. (1933). Possible chemical nature of tobacco mosaic virus. *Nature*, Lond., CXXXII, 1003.
- (3) BEIJERINCK, M. W. (1898). Ueber ein Contagium Vivum fluidum als Ursache der Fleckenkrankheit der Tabaksblätter. *Verh. Akad. Wet., Amst., II*, Ser. 6 (5).
- (4) BREWER, P. H. and KRAYBILL, H. R. *et al.* (1930). Purification and certain properties of the virus of typical tomato mosaic. *Phytopathology*, xx (12), 943-50.
- (5) BRONFENBRENNER, J. J. (1930). Studies in the nature of the Bacteriophage. *J. Bact.* xix, 14.
- (6) CALDWELL, J. (1930). The Physiology of virus diseases in plants. I. The movement of mosaic in the tomato plant. *Ann. appl. Biol.* xvii, 429-43.
- (7) — (1933). The Physiology of virus diseases in plants. IV. The nature of the virus agent of aucuba or yellow mosaic of tomato. *Ibid.* xx, 100-16.
- (8) — (1934). Possible chemical nature of tobacco mosaic virus. *Nature*, CXXXIII, 177.
- (9) FREIBERG, G. W. (1917). Studies on the mosaic diseases of plants. *J. Miss. Bot. Gard.* iv, 175-232.
- (10) KLIGLER, I. J. and OLITSKY, L. (1931). Studies on protein-free suspensions of viruses. *Brit. J. exp. Path.* xii, 393.
- (11) KREUGER, A. P. and TAMADA, H. T. (1929). The preparation of relatively pure bacteriophage. *J. ger. Phys.* xiii, 145.
- (12) LOJIN, M. and VINSON, C. G. (1931). Effect of enzymes upon the infectivity of the virus of tobacco mosaic. *Contr. Boyce Thompson Inst.* iii, 147-62.
- (13) OPPENHEIM, C. (1925). *Die Fermente*. 2 vols. Leipzig.
- (14) PIRIE, A. (1931). Adsorption experiments with the Rous sarcoma virus. *Brit. J. exp. Path.* xii, 373-8.
- (15) SMITH, J. HENDERSON (1928). Experiments with a mosaic disease of tomato. *Ann. appl. Biol.* xv, 155-167.
- (16) VINSON, G. C. (1927). Precipitation of the virus of tobacco mosaic. *Science*, LXVI, 357-8.
- (17) VINSON, G. C. and PETRE, A. W. (1929). Mosaic disease of tobacco. *Bot. Gaz.* LXXXVII, 14-38.
- (18) — (1931). Mosaic disease of tobacco. (II) Activity of the virus precipitates of lead acetate. *Contr. Boyce Thompson Inst.* iii, 131-45.
- (19) WOODS, A. F. (1902). Observations on the mosaic disease of tobacco. *Bull. U.S. Bur. Pl. Ind.* xviii, 1-24.



Fig. 1.

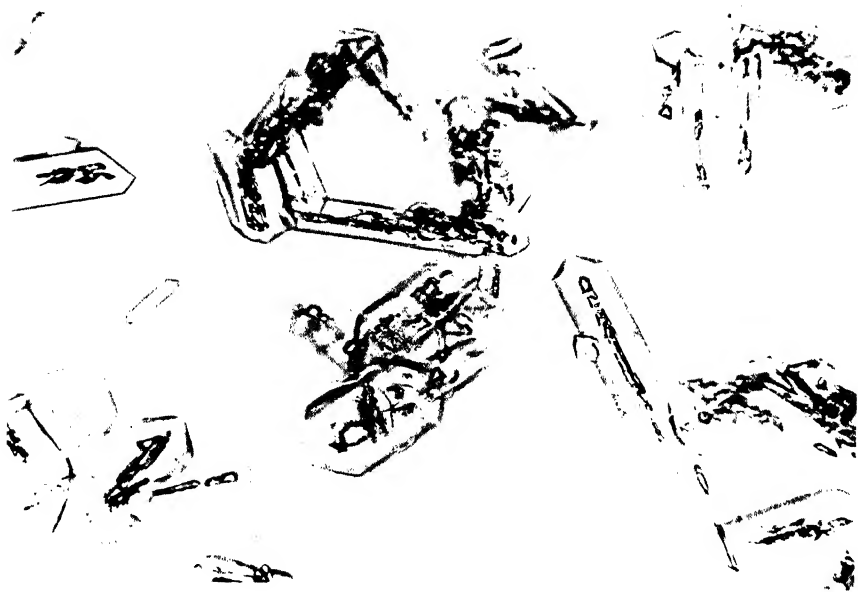


Fig. 2.

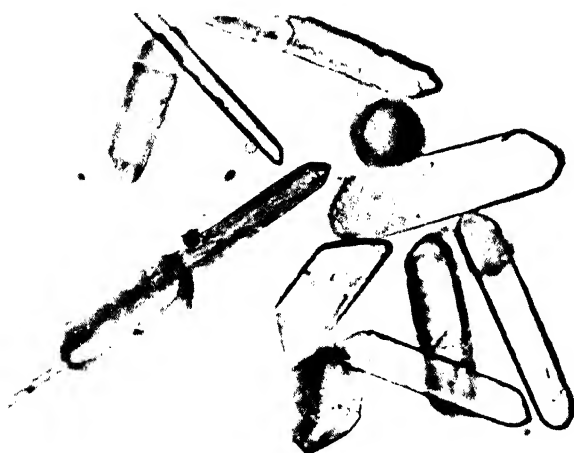


Fig. 3.

EXPLANATION OF PLATES IX AND X.

PLATE IX.

Fig. 1. Leaves of *Nicotiana glutinosa* plants inoculated with (1) treated juice; (2) basic lead acetate supernatant; (3) first KH_2PO_4 eluate; (4) second KH_2PO_4 eluate; (5) first K_2HPO_4 eluate; (6) second K_2HPO_4 eluate; (7) third K_2HPO_4 eluate; (8) precipitate + water. For details see text.

PLATE X.

Fig. 2. Crystals from $M/15$ KH_2PO_4 solution with virus or healthy juice added (precipitated with acetone).

Fig. 3. Crystals from $M/15$ KH_2PO_4 solution, precipitated with acetone.

(Received July 6th, 1934.)

[FROM THE ANNALS OF APPLIED BIOLOGY, VOL. XXI, No. 4,
pp. 566-580, NOVEMBER, 1934.]

[*All rights reserved*]

A COMPARISON OF ENGLISH AND CANADIAN TOMATO VIRUS DISEASES

BY G. C. AINSWORTH

(Experimental and Research Station, Cheshunt, Herts.),

G. H. BERKELEY

*(Laboratory of Plant Pathology, St Catharines, Ontario, Canada, on
exchange basis for a year at East Malling Research Station,
East Malling, Kent, England),*

AND J. CALDWELL

(Department of Plant Pathology, Rothamsted Experimental Station).

(With Plates XXVI and XXVII.)

INTRODUCTION.

IN recent years a considerable literature dealing with tomato virus diseases, especially those of the "streak" type, has been published both in America and in this country, but as no comparative study of the diseases had been made considerable doubt existed whether certain diseases with a common symptom picture, which occur in both countries, were in reality identical from a causal standpoint. Still further confusion had resulted from the fact that tomato streak has been found in England to be caused, in the majority of cases, by a single virus, whereas in America a disease with identical symptoms was considered to be caused by a mixture of a potato and a tobacco virus. The whole question, therefore, of the virus diseases of tomato seemed to need clarifying, and it was with this idea in mind that the present comparative study was started in July 1933, when it was possible to have the collaboration of G. H. Berkeley who was on a year's visit to this country. The work here recorded has been carried out by the three authors in their respective laboratories and notes have been compared from time to time. In this way it was hoped to eliminate errors due to any accidental infections, and the conclusions, while agreed upon by the three authors in collaboration, have been arrived at by each independently. It was hoped that such a study would enable us to define certain viruses which are known to attack the tomato and to associate them with the diseases found in that crop both in England and in Canada. As a result of this study it has been found that the more common viruses occur in both countries.

Nomenclature.

It is probably desirable at this point to make clear the distinction between a virus and a virus disease. In the host plant a group of abnormal symptoms characterise a disease. The symptoms may be caused by any pathogen or group of pathogens and these in turn may be viruses, if they conform to the recognised requirements. A single *virus* may be responsible for more than one *disease*, *e.g.* tobacco virus No. 1 causes tobacco mosaic in tobacco and tomato mosaic in tomato. A single disease condition, on the other hand, may be caused by different viruses, *e.g.* tomato streak, which may be caused by a single virus or by a mixture of two viruses.

Reference must be made to a paper by Johnson(21) in which he described the properties of nine different tobacco viruses. These viruses, he was able to show, caused distinct diseases in tobacco and had various characteristics which separated them one from another. In this paper Johnson's nomenclature has been employed for the viruses which he described, in so far as they appeared in our material. Other viruses which have been found have been defined as precisely as possible with the view to clarifying the confusion which indisputably exists, not only in the earlier but also in the more recent literature.

In the first part of this paper the tomato mosaics, the streak complex and spotted wilt are defined, the literature cited and the characteristics of the viruses described. In the second part, a detailed description of the results obtained from the examination and comparison of the English and Canadian material is given, together with a discussion of the general problem.

PART I. DESCRIPTION OF THE DISEASES AND THE VIRUSES.

(1) *Tomato mosaic.*

DISEASE SYNONYMS. Ordinary or mild tomato mosaic.

VIRUS. *Tobacco virus* No. 1 (Johnson(21)).

LITERATURE. Tobacco mosaic was the first plant disease caused by a filterable virus to be recognised (Ivanowski, 1892), and Clinton in 1908 first demonstrated that tobacco mosaic could be transmitted to tomato and *vice versa* (see Burnett and Jones(12)). Common tobacco mosaic is caused by Johnson's *tobacco virus* No. 1(21), the type disease being that described by Allard(4). Allard(5) considered the viruses of tobacco mosaic and of tomato mosaic to be identical, and this view has been generally held by later workers (see Dickson(13), Walker(32), Burnett and Jones(12), Ainsworth(2) and others).

DISEASE SYMPTOMS. *Tomato.* Dark green—light green mottle (see Plate XXVI, fig. 2), slight stunting, varying degree of leaf distortion but no necrosis. In winter mottle absent or very slight, but leaf distortion and stunting more severe.

Nicotiana tabacum. No local necrotic lesions, dark green—light green mottle and malformation of leaves. Some stunting and “break” in flower colour.

Nicotiana glutinosa.
Datura Stramonium. } Local necrotic lesions, no systemic infection.

PROPERTIES OF TOBACCO VIRUS No. 1. *Filterability.* Passes Pasteur-Chamberland filters L1–L7.

Resistance to ageing. Survives *in vitro* for many years.

Resistance to heat. Survives 80° C. for 10 min. Destroyed at 90° C. for 10 min.

(2) *Yellow mosaic of tomato.*

DISEASE SYNONYMS. “Aucuba” mosaic of tomato. This disease is quite generally called “aucuba mosaic” of tomato, but, as the name has in the past led to some confusion with the aucuba mosaic of potato, it is suggested that the name yellow mosaic is preferable.

VIRUS. *Tobacco virus No. 6* (Johnson(21)).

LITERATURE. This disease was first described in England by Bewley(8) and later in more detail by Henderson Smith(24), who suggested that the virus responsible was the same as that of yellow mosaic of tobacco. Further work has shown that the two viruses are indistinguishable.

DISEASE SYMPTOMS. *Tomato.* Bright yellow mottle on leaves (see Plate XXVI, fig. 3) and fruit (see Plate XXVII, fig. 9), some leaf distortion and stunting of plant. In winter marked leaf distortion and stunting.

N. tabacum. Local necrotic lesions, bright yellow mottle, some necrosis, malformation, stunting and “break” in flower colour.

N. glutinosa.
D. Stramonium. } Local necrotic lesions. No systemic infection.

There is some evidence of the existence of definite strains of this virus. These are being investigated by one of the present authors (J. C.) and will be dealt with in a later paper.

PROPERTIES OF TOBACCO VIRUS No. 6. *Filterability.* Passes Pasteur-Chamberland filters L1–L7.

Resistance to ageing. Survives several years *in vitro*.

Resistance to heat. Survives 80° C. for 10 min. Destroyed at 90° C. for 10 min.

(3) *Tomato streak.*

LITERATURE. "Streak" disease of tomato was first recorded in America by Lodeman in 1892 and in the same year was described by Bailey as a bacterial disease (see Burnett and Jones(12)). In 1916 streak was attributed by Howitt and Stone(16) to malnutrition, while later (1925) Stone(28) considered the disease to be associated with an excess of nitrogen and a deficiency of potash in the soil and that it could be controlled by increasing the phosphate and potash.

Jackson(17) in 1917 expressed the opinion that streak was a virus disease, and a similar view was held by Gardner and Kendrick(15) who described streak as a form of "tomato mosaic." Dickson(13) attributed streak of Quebec to a double infection with tobacco mosaic and potato mosaic viruses. That streak can be produced in this way has been confirmed by many other American workers including Johnson(20), Berkeley(7), Blood(10), Stover(29), Doolittle and Blood(14), Vanterpool(31), and Burnett and Jones(12), while Valleau and Johnson(30) were able to produce streak in tomato by combining the latent potato virus (see p. 577) with three strains of tobacco mosaic, three strains of etch and three strains of cucumber mosaic. The literature suggests that the tomato streak of America is regarded as being due to a mixed virus infection, though Valleau and Johnson(30) also found a single virus (that of ring mosaic) belonging to the tobacco virus group, which caused streak in tomato, and Johnson(21) described *tobacco virus No. 9* as the cause of "tomato stem-necrosis," a type of streak.

In England, Paine and Bewley(22) attributed "stripe" disease to *Bacillus lathyri*, and later Bewley and Corbett(9) recognised a connection between "stripe" and "mosaic." Jarrett(18) was of the opinion that tomato streak was due to *tobacco virus No. 1* without the participation of potato mosaic, but she also recognised the mixed virus condition and distinguished it as "experimental streak." The position was re-examined by Ainsworth(2), who concluded that a mixed-virus streak was of very rare occurrence in the British Isles; a single virus, distinct from *tobacco virus No. 1*, being responsible for the disease, which, following Jarrett's terminology, he called *glasshouse streak*. A possible explanation of the discrepancies between the results of Ainsworth(2) and those of Jarrett(18) and Bewley and Corbett(9) is that the earlier workers used mixtures of tomato mosaic and single-virus streak, for such mixtures (as pointed out(2), p. 425) may remain long undetected if serial transfers are made through tomato.

K. M. Smith⁽²⁷⁾ in his recent survey of the plant viruses described single-virus streak as a type of "mosaic" and suggested that streak in glasshouses was caused by double infection with spotted wilt and tomato mosaic viruses. This latter view is untenable as streak is prevalent on commercial nurseries from which spotted wilt is known to be absent. We think that K. M. Smith has not attached sufficient importance to the existence of a single virus which causes the greater part of the streak under commercial conditions.

NOMENCLATURE. From the above it is at once apparent that there is more than one "streak" disease of tomato, and as a result of this investigation it is proposed to recognise at the present time the four following streak diseases. Although in so far as symptoms are concerned these diseases may be identical, nevertheless they have been proved to be caused by different viruses. They are:

- (i) Single-virus streak;
- (ii) Mixed-virus streak;
- (iii) Stem-necrosis streak;
- (iv) Ring mosaic streak.

(i) *Single-virus streak.*

DISEASE SYNONYMS. Glasshouse streak or "stripe." We suggest that both these synonyms be dropped. The first because this disease is a trouble of outdoor as well as of glasshouse tomatoes and the second because "stripe," which was first used by Bewley in connection with a bacterial disease exhibiting streak-like symptoms, should be used only in the original sense, that is, in connection with *Bacillus lathyri*, as the cause of stripe disease.

VIRUS. *Tomato streak virus No. 1* (this paper).

DISEASE SYMPTOMS. *Tomato.* Necrotic lesions on stem, leaves and fruit, mottle, some leaf distortion and stunting of plant (see Plate XXVII, figs. 5 and 6). Under certain conditions the disease may manifest itself as a mottle only.

N. tabacum. Local necrotic lesions—no other symptoms or systemic necrosis, mottle and stunting.

<i>N. glutinosa.</i> <i>D. Stramonium.</i>	}	Local necrotic lesions, no systemic infection.
---	---	--

PROPERTIES OF TOMATO STREAK VIRUS No. 1. *Filterability.* Passes Pasteur-Chamberland filters L1-L7.

Resistance to ageing. Survives *in vitro* several years.

Resistance to heat. Survives 80° C. for 10 min. Destroyed by 90° C. for 10 min.

(ii) *Mixed-virus streak.*

DISEASE SYNONYMS. Experimental streak, winter blight.

VIRUS. A mixture of two or more viruses. Generally this mixture is a tobacco mosaic virus or *tomato streak virus* No. 1, and a potato virus of the *X* type (*Potato virus X*, see Smith(25)).

DISEASE SYMPTOMS. *Tomato.* Necrotic lesions on stem, leaves and fruit, mottle, some leaf distortion and stunting of plant. A mottle without any necrosis is rare (cf. single-virus streak above).

N. glutinosa. } Local lesions followed by systemic symptoms
D. Stramonium. } caused by the potato virus fraction.

These host plants are useful indicators for streaks of the mixed-virus type, since the potato fraction of such mixtures produces systemic symptoms in them.

PROPERTIES OF VIRUSES OF THE TOBACCO MOSAIC GROUP. See above.

PROPERTIES OF POTATO MOSAIC VIRUS (*X* TYPE). *Filterability.* Passes L3 but not usually L5 or L7 Pasteur-Chamberland filters.

Resistance to ageing. Survives *in vitro* six months or less.

Resistance to heat. Survives 60° C. for 10 min. Destroyed by 70° C. for 10 min.

(iii) *Stem-necrosis streak*, see Part II, p. 576.

(iv) *Ring mosaic streak*, see Part II, p. 576.

(4) *Spotted wilt of tomato.*

VIRUS. *Spotted wilt* or "*T.S.W.*" virus (type Samuel *et al.*(23)).

LITERATURE. This disease was first noted by Brittlebank(11) in Australia. The type disease was described by Samuel *et al.*(23), and the description amplified by Bald and Samuel(6). K. M. Smith(26) first detected the disease in England and his findings have been confirmed by Ainsworth(1) and others.

DISEASE SYMPTOMS. *Tomato.* First symptoms a bronzing of the apical leaves (see Plate XXVII, fig. 7) and stunting, later a yellow mottle of the older leaves. Fruit marked (see Plate XXVII, fig. 8). Lesions rarely found in the stem or petioles; cf. streak above.

N. tabacum. Local necrotic lesions, systemic necrosis, stunting.

N. glutinosa. Local necrotic lesions, systemic necrosis, stunting.

D. Stramonium. Local necrotic lesions. Systemic symptoms: mottle, leaf distortion and stunting of plant.

PROPERTIES OF SPOTTED WILT VIRUS. *Filterability.* Cannot be filtered through Pasteur-Chamberland filters.

Resistance to ageing. Survives *in vitro* only a few hours.

Resistance to heat. Destroyed at 45° C. for 10 min.

This virus can attack a large number of different plants and is widely distributed.

Insect vectors.

At the present time no insect vectors of *tobacco virus No. 1*, *tobacco virus No. 6*, *tomato streak virus No. 1* or *potato virus X* are known.

Spotted wilt virus is very efficiently spread by *Thrips tabaci* Lind. in this country and by the allied insect *Frankliniella insularis* Franklin in Australia (see (26) and (23)).

The preliminary differentiation of the common viruses of the tomato.

It can be seen from the descriptions of the viruses that the five most commonly occurring types can be readily separated from each other by their reactions on two host plants, *N. tabacum* and *N. glutinosa*.

Table I summarises the relevant details for the preliminary differentiation of the viruses of common tobacco and tomato mosaic, potato mosaic, yellow mosaic of tomato, single-virus streak and spotted wilt.

Table I.

Differentiation of the common tomato viruses.

Virus of	No local lesions but systemic chlorosis in <i>N. tabacum</i>	Systemic chlorosis in <i>N. glutinosa</i>	Local lesions and systemic chlorosis in <i>N. tabacum</i>	Local lesions and either no systemic symptoms or systemic necrosis in <i>N. tabacum</i>	No symptom after 24 hours' exposure of juice
Tomato mosaic (common tobacco mosaic)	+	—	—	—	—
Potato mosaic	—	+	—	—	—
Yellow mosaic of tomato	—	—	+	—	—
Single-virus streak	—	—	—	+	—
Spotted wilt	—	—	—	—	+

PART II. EXAMINATION OF THE CANADIAN MATERIAL.

The samples of the Canadian material examined were collected by G. C. Chamberlain, Laboratory of Plant Pathology, St Catharines, Ontario, and by W. Newton, Laboratory of Plant Pathology, Saanichton, British Columbia, to whom we tender our best thanks. The material sent was considered by the collectors to be quite typical of the disease condition it represented.

The material was sent to this country in a dried state, and on arrival each sample was divided, portions being independently examined at the

Cheshunt Experimental Station, the East Malling Research Station and Rothamsted Experimental Station. All the samples were examined at two of these stations and most at all three, so that the findings of any one observer were readily confirmed by the others. There was general agreement among the collaborators, and the results presented are, unless stated to the contrary, based on the work of all three authors.

The different viruses found were tested on a range of host plants over a period of nearly a year and were compared with the standard material of this country.

Methods. The method of inoculation was to macerate the diseased tissue in water in a mortar, and to rub the leaves of the test plant gently with a plug of cotton-wool or muslin soaked in the inoculum thus prepared.

As the object of the investigation was to compare English and Canadian tomato virus diseases it seemed advisable to test the viruses concerned on both English and Canadian varieties of the host. Accordingly the common Canadian tomato variety Grand Rapids was used at East Malling, and the English varieties Kondine Red and E.S. 1 at Rothamsted and Cheshunt respectively. White Burley tobacco and a Virginian tobacco and the same strain of *Nicotiana glutinosa* were used by all three investigators as the differential hosts.

The plants were grown in individual pots, inoculated when from 3 to 6 in. high, and were usually kept under observation for a further 4-5 weeks.

All tests of the effects of the viruses on the different host plants were carried out several times at each station in order to obtain the symptom picture under both summer and winter growing conditions.

Experimental results.

Table II summarises the details of the material examined and the viruses isolated. The diseased material, as received, was inoculated into tomato, tobacco and *N. glutinosa* plants and the virus or viruses present identified. Table III summarises the symptoms obtained and other details of the experiments.

The viruses found in the Canadian material.

(a) Canadian tomato mosaic.

Four samples (Table II, *a*, *b*, *c*, *k*) labelled tomato mosaic were received, and on examination two (*a* and *c*) proved to be true tomato mosaic (*i.e.* caused by *tobacco virus No. 1*), while the other two (*b* and *k*) were found to contain the virus of single-virus streak (*i.e.* *tomato streak*).

Table II.
List of material with viruses found.

Canadian material:		Viruses obtained
(a) Tomato mosaic	Vineland, Ontario (tomato var.* Riverside Favourite)	<i>Tobacco virus</i> No. 1
(b) Tomato mosaic	Stamford, Ontario	<i>Tomato streak virus</i> No. 1
(c) Tomato mosaic	Vineland, Ontario	<i>Tobacco virus</i> No. 1
(d) Tomato streak (7 samples)	Stamford, Ontario	<i>Tomato streak virus</i> No. 1
(e) Tomato streak	Grimsby, Ontario	<i>Tobacco virus</i> No. 1
(f) Tomato streak	Beamsville, Ontario	<i>Tomato streak virus</i> No. 1
(g) Tomato streak	St Catharines, Ontario (tomato var. Sutton's Favourite)	<i>Tobacco virus</i> No. 1 (<i>Tobacco virus</i> No. 9)
(h) Tomato streak	Niagara Falls, Ontario	<i>Tomato streak virus</i> No. 1 and potato virus X type
(i) Tobacco mosaic	Ontario	<i>Tobacco virus</i> No. 1
(j) Irish Cobbler potato "mosaic"	New Brunswick	Potato virus X type
(k) Tomato mosaic	British Columbia	<i>Tomato streak virus</i> No. 1
(l) Yellow tomato mosaic	British Columbia	<i>Tobacco virus</i> No. 6
(m) Tomato streak	British Columbia	<i>Tomato streak virus</i> No. 1 and potato virus X type
(n) Tomato streak	British Columbia	<i>Tomato streak virus</i> No. 1
(o) Tomato streak	British Columbia	<i>Tomato streak virus</i> No. 1
(p) Ring mosaic	Kentucky, U.S.A.	<i>Ring mosaic virus</i>
English material:		
(q) Tomato mosaic	Cheshunt	<i>Tobacco virus</i> No. 1
(r) Tobacco mosaic	Rothamsted (authentic material from Johnson)	<i>Tobacco virus</i> No. 1
(s) Tomato streak	Cheshunt	<i>Tomato streak virus</i> No. 1
(t) Tomato spotted wilt	Cheshunt	<i>Spotted wilt virus</i>
(u) Yellow tomato mosaic	Rothamsted (authentic material of Henderson Smith)	<i>Tobacco virus</i> No. 6

* All Canadian tomato material was Grand Rapids variety unless stated otherwise.

Table III.
Details of plants inoculated and symptoms.

	Tomato	<i>N. tabacum</i>	<i>N. glutinosa</i>	Virus
Canadian material:				
(a) Tomato mosaic	M. (12)*	M., m. (9)	l.l. (6)	Tb.v.1
(b) Tomato mosaic	M. or M. S. (12)	l.l., S. (9).	l.l. (6)	T.st.v.1
(c) Tomato mosaic	M. (52)	M., m. (27)	l.l. (14)	Tb.v.1
(d) Tomato streak (all samples)	M. or M. S. (63)	l.l., S. (33)	l.l. (14)	T.st.v.1
(e) Tomato streak	M. (52)	M., m. (37)	l.l. (17)	Tb.v.1
(f) Tomato streak	M. or M. S. (49)	l.l., S. (21)	l.l. (8)	T.st.v.1
(g) Tomato streak	(M. M. or S. (64)	M., m. M. (mild) (47)	l.l. (14)	(Tb.v.1 {Tb.v.9
(h) Tomato streak	S. (36)	l.l., S. (34)	l.l., M. (17)	{T.st.v.1 {P.v.X
(i) Tobacco mosaic	M. (34)	M., m. (25)	l.l. (18)	Tb.v.1
(j) Irish Cobbler potato mosaic	M. (mild)† (20)	M. (mild) (7)	M. (6)	P.v.X
(k) Tomato mosaic	M. or M. S. (23)	l.l., S. (9)	l.l. (10)	T.st.v.1
(l) Yellow tomato mosaic	M. (6)	l.l., M., S. (3)	l.l. (2)	Tb.v.6
(m) Tomato streak	S. (9)	l.l., S. (5)	l.l., M. (3)	{T.st.v.1 {P.v.X
(n) Tomato streak	M. or M. S. (15)	l.l., S. (10)	l.l. (8)	T.st.v.1
(o) Tomato streak	M. or M. S. (6)	l.l., S. (4)	l.l. (2)	T.st.v.1
(p) Ring mosaic	S. (32)	l.l., S. (18)	l.l. (18)	Ring mosaic virus
English material:				
(q) Tomato mosaic	M. (19)	M., m. (9)	l.l. (7)	Tb.v.1
(r) Tobacco mosaic	M. (17)	M., m. (8)	l.l. (2)	Tb.v.1
(s) Tomato streak	M. or M. S. (19)	l.l., S. (9)	l.l. (6)	T.st.v.1
(t) Spotted wilt	Bronzing (27)	l.l., S. (22)	l.l., S. (12)	Spotted wilt virus
(u) Yellow tomato mosaic	M. (20)	l.l., M., S. (12)	l.l. (12)	Tb.v.6

M. = mottle without necrosis.

m. = malformation.

S. = streak on tomato, necrosis on
tobacco or *N. glutinosa*.

l.l. = local lesions.

Tb.v.1 = *Tobacco virus* No. 1.

Tb.v.6 = *Tobacco virus* No. 6.

Tb.v.9 = *Tobacco virus* No. 9.

T.st.v.1 = *Tomato streak virus* No. 1.

P.v.X = potato virus X type.

* Numbers within brackets denote total numbers of plants infected to study the symptom pictures.

† Also slight necrosis.

virus No. 1), and they presumably, when collected, showed the mottle symptom only and were therefore considered to be mosaic.

The viruses of the two types were compared with the respective standard strains, *tobacco virus* No. 1 (*r*) and *tomato streak virus* No. 1 (*s*), as regards differential hosts, filterability and resistance to heat, and they were found to be identical with them. For differential host reactions and properties of these viruses see pp. 568 and 570.

(b) Canadian yellow mosaic of tomato.

One sample only (*k*), from British Columbia, was examined, and this proved to be identical in all respects with the English material (*u*) when compared at East Malling and Rothamsted. For differential host reactions and properties of this virus (*tobacco virus* No. 6), see p. 568.

(c) Canadian tobacco mosaic.

One sample only (*i*) was obtained from Ontario, and on examination the virus proved to be identical in all respects with *tobacco virus* No. 1. For differential host reactions and properties of this virus see p. 568.

(d) Canadian tomato streak material.

In all, fourteen samples of tomato streak were obtained of which ten (seven of *d*, *f*, *m* and *o*) proved to be single-virus streak, *i.e.* caused by *tomato streak virus* No. 1. This is the first record of single-virus streak in Canada. For differential host reactions and properties of this virus see p. 570.

Two samples, one from Niagara Falls, Ontario (*h*) and the other from British Columbia (*m*) proved to be mixed-virus streak, the components in both cases being *tomato streak virus* No. 1 and a potato virus of the *X* type. For differential host reactions and properties of these viruses see pp. 570 and 571.

In one sample (*e*), though the diseased material as received showed distinct necrotic lesions on the stem and petioles, only *tobacco virus* No. 1 was detected. The explanation is probably that in this instance the disease was a streak of the mixed-virus type and that the potato virus fraction did not survive the transportation period; otherwise it is difficult to account for the streak lesions on the original material.

One sample from St Catharines (*g*) showed streak symptoms. At Cheshunt and Rothamsted the only virus found in this material was *tobacco virus* No. 1, while at East Malling a virus thought to resemble *tobacco virus* No. 9 (the cause of stem-necrosis of tomato, Johnson⁽²¹⁾) was isolated. This appears to be the first record of the occurrence of

tobacco virus No. 9, which has not so far been isolated from English material, since Johnson originally defined it in 1927 (21). The differential host reactions and properties of this virus may well be detailed here.

Stem-necrosis streak.

VIRUS. *Tobacco virus* No. 9 (Johnson (21)).

DISEASE SYMPTOMS. *Tomato*. Mottle on leaves with necrosis on petioles and stems.

N. tabacum. No local lesions on rubbed leaves. Faint systemic mottle without stunting or distortion.

N. glutinosa.
D. Stramonium. } Local necrotic lesions. No systemic infection.

PROPERTIES OF TOBACCO VIRUS No. 9. *Filterability*. Passes Pasteur-Chamberland filters L1-L7.

Resistance to ageing. Survives 3 months or more (Johnson (21)).

Resistance to heat. Survives 80° C. for 10 min. Destroyed at 90° C. for 10 min.

Ring mosaic streak of tomato.

This disease was not actually found among the plant material from commercial nurseries of England or Canada, but a sample was kindly sent to us by E. M. Johnson of Kentucky. For the first few months the material was examined at Rothamsted and later some was sent to East Malling and Cheshunt. This disease of tomato has been described by Valleau and Johnson (30) and by Johnson (19), but the virus which causes it has not yet been described. The properties and host reactions of the virus have been determined and are appended.

Ring mosaic streak.

VIRUS. *Ring mosaic virus*.

DISEASE SYMPTOMS. *Tomato*. Occasional yellow areas on leaves, with little mottle or distortion. Necrotic lesions irregularly distributed on stem, petioles and leaves of older parts of the plant (see Plate XXVI, fig. 4). Little stunting. Under our conditions we did not get the very severe foliage symptoms described by E. M. Johnson (19) for this virus on tomato but the fruit was seriously affected. Green fruit exhibited chlorotic or necrotic areas and ripe fruit was disfigured by the irregular development of red pigment in the skin.

N. tabacum. Local lesions followed by systemic chlorosis and some necrosis—often ring-like.

N. glutinosa.
D. Stramonium. } Local necrotic lesions, no systemic infection.

PROPERTIES OF RING MOSAIC VIRUS. *Filterability.* Passes Pasteur-Chamberland filters L1-L7.

Resistance to ageing. Survives some years in dry tissues.

Resistance to heat. Survives 70° C. for 10 min. Destroyed at 80° C. for 10 min.

Irish Cobbler potato mosaic.

Infected Irish Cobbler potato tubers from New Brunswick were sprouted at East Malling and leaves sent to Cheshunt. The virus isolated at both East Malling and Cheshunt was found to be a potato virus of the X type.

POTATO VIRUSES.

Burnett and Jones⁽¹²⁾ in their investigation of the effects of certain potato viruses on tomato plants distinguished two types of the "latent" potato virus, viz. the "latent" and the "virulent latent." Both these types have been studied during the present work.

From the various samples of Canadian tomato material three isolations of potato viruses were made. Two isolated at Cheshunt approximated to the "latent" virus while one isolated at East Malling was nearer the "virulent latent." In an attempt to establish the identity of these viruses they, together with the Irish Cobbler potato virus, were compared at Cheshunt with authentic *potato virus X*, kindly supplied by K. M. Smith, and several other strains of the X virus. As the result of this comparison, a more detailed account of which has been given by Ainsworth⁽³⁾, it was concluded that all these viruses are potato viruses of the X type differing from each other merely in virulence.

These viruses are filterable through L1 and L3 Pasteur-Chamberland filters but not usually through L5 and L7, and are inactivated by heating to 70° C. for 10 min. The symptom picture on tomato varies from a mild or almost imperceptible leaf-mottle to a well-defined leaf-necrosis (especially of the older leaves) without lesions on the petioles or stem.

Each of these potato viruses has been mixed with *tobacco virus No. 1* and inoculated into tomato. Streak resulted in every case, though it was interesting to note that the intensity of the streak symptoms varied, the symptoms being most intense when the most virulent potato virus was used.

DISCUSSION AND CONCLUSIONS.

The results reported above show that the tomato mosaic, common tobacco mosaic, yellow mosaic of tomato, single-virus streak and mixed-virus streak diseases which occur in both England and Canada are caused by the same viruses or virus mixtures.

Of the fourteen samples of Canadian streak material examined two were found to be of the mixed-virus type, and from one sample *tobacco virus No. 1* only was isolated. The presumption is that in this latter material, the potato virus fraction if present had been lost during drying and transportation. This would suggest that a mixed-virus streak of the tobacco-potato mosaic virus type is more common in Canada than it is in England where it is rarely found. Although the results obtained agree in this particular with the view expressed in Canadian literature that a mixed-virus streak of this type occurs under commercial conditions in Canada, they tend, in the main, to indicate that the usual cause of streak is the single virus which is commonly found under English conditions. This virus appears to be as often found in Canadian material as in English. Of the two samples of mixed-virus streak in which both components of the mixture were isolated the mixture was not the classical one of *tobacco virus No. 1* and a potato mosaic virus, but *tomato streak virus No. 1* and a potato mosaic virus of the X type. As has been shown, the former virus of itself causes streak in many cases but may induce a disease of the mosaic type, without necrosis. Combined with *potato virus X*, however, it produces, invariably, definite streak lesions.

Ring mosaic virus and *tobacco virus No. 9* cause distinct diseases which have not so far been recorded as occurring in England.

SUMMARY.

It has been found from a comparative study of English and Canadian tomato virus diseases that the commoner viruses which affect the tomato occur in both countries.

Tomato streak caused by the same single virus occurs in both countries, but streak due to a mixed-virus infection appears to be more frequent in Canada than in England.

The following diseases with their causal viruses are described in this paper. Tomato mosaic (*tobacco virus No. 1*), yellow mosaic of tomato (*tobacco virus No. 6*), single-virus streak (*tomato streak virus No. 1*), mixed-virus streak (a tobacco virus + a potato virus) and spotted wilt (*spotted wilt virus*). The presence of the last-named disease in Canada was not established.

Ring mosaic virus and *tobacco virus No. 9*, which have not been recorded in England, are also described.

ACKNOWLEDGMENT.

We wish to thank Dr W. F. Bewley and Dr J. Henderson Smith for reading through the manuscript, and Dr G. H. Pethybridge for advice and help. G. H. Berkeley especially wishes to acknowledge the great assistance given to him at East Malling by the installation of a system of artificial lighting, which enabled him to carry out successfully his part of this co-operative work.

REFERENCES.

- (1) AINSWORTH, G. C. (1933). Spotted wilt of tomatoes. *Ann. Rep. Exper. and Res. Sta. Cheshunt, Herts.* xviii, 39-42.
- (2) — (1933). An investigation of tomato virus diseases of the mosaic, "stripe," streak group. *Ann. App. Biol.* xx, 421-8.
- (3) — (1934). A comparison of certain English and Canadian potato viruses found infecting tomatoes. *Ibid.* xxi, 581-7.
- (4) ALLARD, H. A. (1914). The mosaic disease of tobacco. *U.S. Dept. Agric. Bull.* No. 40.
- (5) — (1916). The mosaic disease of tomatoes and petunias. *Phytopath.* vi, 328-35.
- (6) BALD, J. G. and SAMUEL, G. (1931). Investigations on "spotted wilt" of tomatoes. II. *Council Sci. Ind. Res. Australia Bull.* No. 54.
- (7) BERKELEY, G. H. (1927). Studies in tomato streak. *Sci. Agric.* vii, 210-23.
- (8) BEWLEY, W. F. (1923). Mosaic disease of the tomato. *Ann. Rep. Exper. and Res. Sta. Cheshunt, Herts.* ix, 67-9.
- (9) BEWLEY, W. F. and CORBETT, W. (1927). Mosaic disease of the tomato. *Ibid.* xiii, 51-9.
- (10) BLOOD, H. L. (1928). A "streak" disease of tomato produced by a disturbing principle from apparently healthy potatoes in combination with tomato mosaic virus. *Phytopath.* xviii, 311.
- (11) BRITTLEBANK, C. C. (1919). A new tomato disease—"spotted wilt." *Journ. Agric. Vict.* xxvii, 231-5.
- (12) BURNETT, G. and JONES, L. K. (1931). The effect of certain potato and tobacco viruses on tomato plants. *Washington Agric. Exper. Sta. Bull.* No. 259.
- (13) DICKSON, B. T. (1925). (2) Streak of tomato in Quebec, a "double virus" disease. *Science*, lxii, 398.
- (14) DOOLITTLE, S. P. and BLOOD, H. L. (1930). Investigations of tomato streak. *Abst. in Phytopath.* xx, 134.
- (15) GARDNER, M. W. and KENDRICK, J. B. (1922). Tomato mosaic. *Indiana Agric. Exper. Sta. Bull.* No. 261.
- (16) HOWITT, J. E. and STONE, R. E. (1916). A troublesome disease of winter tomatoes. *Phytopath.* vi, 162-6.
- (17) JACKSON, H. S. (1917). Tomato diseases. *Indiana Agric. Exper. Sta. Ann. Rep.* xxx, 23.
- (18) JARRETT, P. H. (1930). Streak—a virus disease of tomato. *Ann. App. Biol.* xvii, 248-59.
- (19) JOHNSON, E. M. (1930). Virus diseases of tobacco in Kentucky. *Kentucky Agric. Exper. Sta. Bull.* No. 306.

- (20) JOHNSON, J. (1925). Transmission of viruses from apparently healthy potatoes. *Wisc. Agric. Exper. Sta. Res. Bull.* No. 63.
- (21) — (1927). The classification of plant viruses. *Ibid.* No. 76.
- (22) PAINE, S. G. and BEWLEY, W. F. (1919). Studies in bacteriosis. IV. "Stripe" disease of tomato. *Ann. App. Biol.* vi, 183-202.
- (23) SAMUEL, G., BALD, J. G. and PITTMAN, H. A. (1930). "Spotted wilt" of tomatoes. *Council Sci. Ind. Res. Australia Bull.* No. 44.
- (24) SMITH, J. HENDERSON (1928). Experiments with a mosaic disease of tomato. *Ann. App. Biol.* xv, 155-67.
- (25) SMITH, K. M. (1931). The composite nature of certain potato viruses. *Proc. Roy. Soc. B*, cix, 251-67.
- (26) — (1932). Studies on plant virus disease. IX. Further experiments with a ring-spot virus: its identification with spotted wilt of the tomato. *Ann. App. Biol.* xix, 305-30.
- (27) — (1933). *Recent Advances in the Study of Plant Viruses*, pp. xii + 423. London: Churchill.
- (28) STONE, R. E. (1925). Winter blight or streak of tomatoes. Abst. in *Phytopath.* xv, 300.
- (29) STOVER, W. G. (1928). Experiments with tomato streak. Abst. *ibid.* xviii, 154.
- (30) VALLEAU, W. D. and JOHNSON, E. M. (1930). Possible causes of streak in tomato. *Phytopath.* xx, 831-9.
- (31) VANTERPOOL, T. C. (1930). Streak or winter blight of tomato in Quebec. *Phytopath.* xvi, 311-31.
- (32) WALKER, M. N. (1926). A comparative study of mosaic disease of cucumber, tomato and *Physalis*. *Phytopath.* xvi, 431-58.

EXPLANATION OF PLATES XXVI AND XXVII.

PLATE XXVI.

Fig. 1. Healthy tomato leaf (var. E.S. 1).

Fig. 2. Tomato leaf (var. E.S. 1) showing symptoms of tomato mosaic.

(The leaves illustrated in Figs. 1 and 2 are reproduced on the same scale and were corresponding leaves from plants of the same age grown under the same conditions.)

Fig. 3. Tomato leaf showing symptoms of yellow mosaic of tomato.

Fig. 4. Tomato leaf showing symptoms of ring mosaic streak.

PLATE XXVII.

Fig. 5. Tomato plant showing symptoms of single-virus streak.

Fig. 6. Green tomato fruit showing markings caused by *tomato streak virus* No. 1 (single-virus streak).

Fig. 7. Tomato leaves showing bronzing symptom of spotted wilt.

Fig. 8. Green tomato fruit showing symptoms of spotted wilt.

Fig. 9. Ripe tomato fruit showing yellow markings caused by *tobacco virus* No. 6 (yellow mosaic of tomato).

(Received July 4th, 1934.)



Fig. 1.



Fig. 2.



Fig. 3.



Fig. 4.



Fig. 5.



Fig. 7.



Fig. 8.



Fig. 6.



Fig. 9.

[FROM THE ANNALS OF APPLIED BIOLOGY, Vol. XXI, No. 3,
pp. 430-453, August, 1934.]

[*All rights reserved.*]

EXPERIMENTS BEARING ON THE NATURE OF INTRACELLULAR INCLUSIONS IN PLANT VIRUS DISEASES

BY F. M. L. SHEFFIELD, PH.D., F.L.S.

(*Department of Plant Pathology, Rothamsted Experimental
Station, Harpenden, Herts.*)

(With Plates XXIII–XXV.)

CONTENTS.

	PAGE
Introduction	430
Intracellular inclusions in virus diseases	430
Aucuba mosaic disease	431
Hy. III disease	432
Tobacco mosaic disease	433
Attempts to induce changes in the colloids of the plant cell	434
Experimental	436
Attempts to simulate virus inclusion bodies in uninfected cells	436
Material	436
Methods	437
Results	439
Attempts to inhibit the development of inclusion bodies in virus-diseased plants	447
Methods	447
Experiments with Solanaceae	447
Experiments with <i>Cucumis sativus</i>	448
Discussion	449
Summary	451
References	452
Explanation of Plates XXIII–XXV	453

INTRODUCTION.

INTRACELLULAR INCLUSIONS IN VIRUS DISEASES.

SOME thirty years ago Iwanowski first discovered the curious cytological effects which may be induced by infection of a plant with a virus disease⁽⁹⁾. He then described and figured the peculiar amoeboid bodies which he found in the cells of tobacco with mosaic disease. Since that time the presence of similar intracellular inclusions has been reported in

many plants and animals attacked by virus diseases and consequently considerable attention has been attracted to them. It is unnecessary here to discuss the literature as it has frequently been summarised (5, 8, 17), and a full list of papers is given by K. M. Smith (18). Some virus diseases appear to be invariably characterised by these bodies whilst other viruses have no visible effect on the cells of the host. For example, inclusions are always found in the cells of plants infected with tobacco mosaic (5), dahlia mosaic (6) and sugar-cane mosaic (10), but could never be found after infection with cucumber mosaic or petunia mosaic (8). Such intracellular inclusions had never been found in healthy plants nor in plants infected with any disease other than a virus. The bodies are protein in nature and show certain apparent similarities in form and behaviour to amoeboid organisms. In fact, the view was frequently advanced that the bodies were actually organisms, that they were the etiological agents of the disease. However, other workers held the view that they are inanimate, being merely pathological effects.

Aucuba mosaic disease.

Some recent work on the intracellular inclusions in solanaceous plants infected with aucuba mosaic disease of tomato, has lent considerable support to this latter conception of their nature (14). By watching under the microscope living cells of infected plants, continually from the time of inoculation until the maturing of the inclusion bodies, these bodies were seen to be formed by the aggregation and fusion of minute particles of protein which appeared in the cytoplasm of the host a few days after infection. An increasing activity of the cell contents is the first effect of the virus to become apparent microscopically. The cytoplasm becomes more conspicuous, it seems to increase in volume and it flows around and about the cell with increasing rapidity. Then minute yellowish particles appear in the cytoplasm which, as it flows, carries them passively about the cell. If these particles are brought together by converging streams of plasma, they fuse. Gradually the particles increase in size, then they aggregate into a few large masses. These masses unite together until practically all the protein material is contained within a single loosely packed, irregularly shaped body. It soon loses its irregular form, becoming more compact as it assumes a roughly spherical outline. It may become vacuolate (Plate XXIV, fig. 4). After some weeks this body crystallises out and the crystals are dissolved into the cell sap.

These observations, whilst not precluding the idea that the virus may be contained within the body, indicate that the main bulk of the

body is formed from the cytoplasm of the host in response to the stimulus of the virus. They further suggest that the body is essentially a coagulated portion of the cytoplasm.

The mode of formation of bodies due to infection with aucuba mosaic disease was watched in a number of hosts. A few differences were noted in the mature body. In *Solanum nodiflorum*, *S. nigrum* and *S. lycopersicum* the body almost always becomes smoothly rounded. In *Nicotiana tabacum*, *N. glauca* and *N. rustica* the body frequently fails to round off, and in *Hyoscyamus niger* it very seldom does so but persists as a large irregularly shaped granular mass. In the above species, infection is systemic. In *Nicotiana glutinosa*, where local lesions are produced by this virus, no intracellular inclusions could be found.

A number of different hosts infected with various virus diseases have been examined cytologically from time to time. No results have been published, but two examples might, with advantage, be briefly summarised here.

Hy. III disease.

Of the several new virus diseases of *Hyoscyamus niger* recently described (7), Hy. II and Hy. IV produce no inclusion bodies. However, Hy. III regularly induces the production of abnormal structures within the cells of the host plant. For the study of the cytological effects, hosts from the same range that was used with aucuba mosaic were tested and a somewhat similar response was obtained suggesting that, although the production of the body is determined by the virus, its character may be slightly modified by the host. Of the three species of *Solanum*, *S. nodiflorum* and *S. nigrum* proved to be immune to Hy. III, but in *S. lycopersicum* systemic infection is accompanied by the production of large spherical cellular inclusions very similar to those induced by aucuba mosaic disease. In both *Nicotiana tabacum* and *Hyoscyamus niger* the response to the two diseases is similar. In the former there is a tendency for the bodies to remain irregular in form and in the latter they very seldom round off. The bodies induced by Hy. III in these three hosts are formed in precisely the same way as those due to aucuba mosaic infection. The first microscopic effect to become obvious is the accelerated streaming of the cytoplasm in which tiny protein particles soon appear. These ultimately all coalesce to form a single large body (Plate XXIII, fig. 3).

Tobacco mosaic disease.

Much work has been done on the cytology of mosaic disease of tobacco, Goldstein in particular having made a very careful and detailed study⁽⁵⁾. Her description of X-bodies¹ would, except in a few details, appear to be equally applicable to the inclusion bodies formed by aucuba mosaic but Goldstein is convinced that the X-bodies "are not products of the metabolism of diseased cells." An extensive study has therefore been made of fixed and living material of plants infected with tobacco mosaic in an attempt to discover any correlation between the X-bodies of tobacco mosaic and the inclusions formed by aucuba mosaic. The range of hosts used included *Nicotiana tabacum*, *Solanum nodiflorum*, *S. nigrum* and *S. lycopersicum*, all of which yielded similar results. The virus used was Johnson's tobacco virus I.

Seen in fixed material, the inclusions induced by tobacco and aucuba mosaic viruses are not unlike, but when seen side by side and in living cells they appear strikingly different. However, their chemical reactions are alike, so that it is not easy to define adequately the differences which seem to lie in the size and consistency of the bodies. X-bodies of tobacco mosaic are usually much smaller than the inclusions of aucuba mosaic disease. They are also far less granular, appearing to have rather the same consistency as the cytoplasm of the cells containing them. In fact, they appear very like a portion of cytoplasm which, although it has no apparent limiting membrane, is able to retain intact its complement of chondriosomes and oil globules. Goldstein described a membrane, but the writer has been unable to observe this in any virus inclusion bodies examined. An apparent membrane is seen in some photographs, but this is merely an optical effect which when observed visually can be eliminated by refocussing the microscope. The X-body is continually carried about the cell in the cytoplasmic stream. It may become distorted through the buffeting of the flowing plasma just as the nucleus may become temporarily misshapen, but, like the nucleus, it appears always to retain its individuality (Plate XXIV, fig. 1). Many infected plants were examined both before and after the appearance of X-bodies, but no evidence could be obtained as to their origin. It was evident that they were not formed by fusion of particles as are aucuba mosaic bodies, but their origin remained obscure, as no transitional stage between an apparently normal cell and one containing an X-body could

¹ The use of the term "X-body" is, in this paper, confined to the amoeboid intracellular inclusions of tobacco mosaic originally so designated by Goldstein.

be found. After persisting for some weeks, these bodies disappear as suddenly as they arise. They do not crystallise as do the bodies of aucuba mosaic disease.

Associated with tobacco mosaic disease is regularly found a second type of inclusion which at first seems to be similar to many of the crystalline bodies found in healthy plants. This is the striate material (Plate XXIV, fig. 1). It consists of large polygonal plates, either hexagonal or irregular, when they may have been formed by the fusion side by side of smaller hexagonal plates. In side view these plates appear rectangular and show faint lines of striation. On treatment with fixatives the striation becomes more distinct. This striate material persists after the disappearance of the X-body. If plants infected with tobacco mosaic disease are kept for a prolonged period, the vivid dark green mottle is lost from the leaves which tend to develop a yellow blotching. At the same time, it is found that the polygonal crystals in the cells are coalescing, ultimately they are all contained within a single mass which becomes rounded and seems to be in all details like the bodies due to aucuba mosaic disease (Plate XXIV, fig. 3).

ATTEMPTS TO INDUCE CHANGES IN THE COLLOIDS OF THE PLANT CELL.

In considering the nature of the intracellular inclusion bodies, the fact that they are confined to plants and animals infected with virus diseases would appear to be peculiarly significant. However, the mode of formation of the inclusions due to aucuba mosaic virus led almost to the conviction that certain of the bodies are merely coagulation products. Thereby was suggested the possibility that the production of morphologically similar bodies in otherwise normal healthy tissue might be induced by judicious treatment with coagulating agents. Some attempts have therefore been made to determine whether, by treating plants with colloidal reagents:

(a) similar abnormal bodies could be induced in the cells of uninfected plants, and

(b) the reverse reaction could be induced in diseased cells either by inhibiting the formation of bodies or by artificially dispersing them after formation.

A considerable amount of work of a similar nature on animal tissues has been carried out by Bancroft and his co-workers (1,2,4). For instance, certain mental disorders appear to be due to abnormal variations in the colloids of the nervous tissue. Variations are in degree of dispersion only

and hence cannot be demonstrated by any histological technique. By use of colloidal reagents, it is possible to induce symptoms in the normal nervous system not unlike many of those of insanity. Certain functional disorders, due to agglomeration of the colloids of the central nervous system, can be overcome by use of peptising agents as can also the effects of certain drugs. The action of anaesthetics is due to reversible coagulation, and this seems to be true of anaesthesia of both animals and plants (3).

To parallel the phenomena produced by virus diseases involves the production of changes very much greater in degree than anything previously brought about experimentally in the living cell. Further, it was desired to coagulate only a fraction of the cytoplasm, allowing the rest to maintain the normal processes of life. There was available very little experimental data on which to base the work so that only "hit or miss" methods could be employed. A large number of substances are known to coagulate the proteins of the cell. In spite of the vast amount which has been written about the physical and chemical properties of living matter, its constitution is still largely enigmatical. Whilst this remains so, the action of any specific reagent cannot be properly understood or explained. Investigators are handicapped both by the minute dimensions of the cell and by the difficulty of preserving the protoplasm in its normal living state whilst under observation and experimentation. In the case of plants, further difficulties are introduced by the impenetrable nature of the cell wall. Thus, existing knowledge is insufficient to indicate the use of any one series of reagents in preference to any other for the present purpose. It was impossible to try all the substances which suggested themselves, and at first a selection could be made only at random. A number of mineral and organic substances, many of which were known to precipitate cytoplasm, were chosen. During the course of the work, the results obtained by the use of one reagent often suggested the employment of other substances which normally show similar reactions. Occasion arose to obtain spectrographic analyses of certain treated plants for comparison with normal and virus diseased ones. Incidentally, differences were found in the amounts of certain elements present in the specimens of *Solanum nodiflorum* infected with aucuba mosaic and in the normal plants. In each case leaves of some half dozen plants were ashed together, but only one series of analyses was made so the differences in the proportions of these elements might be due to individual variation. This observation accounted for the inclusion of salts of aluminium and nickel, among the reagents used.

A further difficulty was encountered in deciding the concentration of the reagent to be used, especially as many of the substances chosen were known to be highly toxic. The ideal method would be the employment of a large range of concentrations of each reagent. This would have occupied much time and glasshouse space and would, of necessity, have greatly reduced the number of reagents it was possible to employ. It was felt that useful results were more likely to accrue by testing a large number of reagents rather scantily than by the trial of a few reagents in far greater detail. Therefore, in the first instance, one purely arbitrary strength of solution of each substance was selected. If this solution proved toxic, then weaker ones were tried. If the results gave any promise, then new experiments were set up with weaker or stronger solutions as appeared expedient. Alternatively, in some cases the number of doses given was varied.

A short note has already been published on this work(16); a more detailed description now follows.

EXPERIMENTAL.

ATTEMPTS TO SIMULATE VIRUS INCLUSION BODIES IN UNINFECTED CELLS.

Material.

Solanaceous hosts have proved very favourable subjects for the study of the cytological effects of virus diseases by vital methods. Where virus inclusion bodies are found, they are usually most evident in the tegumentary tissues of the leaves. If the latter bear protective hairs then the bodies are usually very abundant in them. Trichomes can be examined microscopically with great simplicity whilst still living. Several of the Solanaceae have hairy leaves and their cells are usually free from ergastic substances which might complicate a study of abnormal inclusions. *Solanum nodiflorum* is particularly useful, for, not only are the cells very clean and clear, but the whole trichome is invested by a thick cell wall. For examination, a strip is cut from the edge of the leaf and is mounted on a slide in water or in isotonic sugar solution. The thick wall delays penetration by the mounting medium for some hours, so that the same cell can be kept under observation for prolonged periods.

S. nodiflorum was used in every experiment in this study. *S. lycopersicum*, *Nicotiana tabacum* and *Hyoscyamus niger* were used for comparison from time to time.

All observations were made on living material. The cells of trichomes were always extensively examined. Occasionally mounts were made of the epidermis by stripping it from the leaf and mounting in an isotonic solution. Hand sections were also occasionally used. In such work no great reliance could be placed on observations on epidermal mounts or hand sections as the cells very soon die and also the mounting medium penetrates rapidly, bringing about abnormal conditions almost immediately. These methods were used only for comparison with the observations made on unsectioned material.

Methods.

Owing to differences in general structure and largely to the nature of the cell wall, the use of vital methods in cytological study is notoriously beset by many more difficulties in plants than in animals. In the work on anaesthesia in plants the usual method of approach is through spraying of the plants. This method was tried but with not much success. Some coagulation, visible microscopically, was obtained, but it was not possible to increase the dose to give greater coagulation without injury to the tissues. More drastic measures had to be employed, the following methods being used.

(1) *Soil treatment.*

The plants were grown in our usual compost: this is grass turves and stable manure stacked for 12-18 months before use, when some sand is added. Pots of approximately 6 in. diameter (32's) were used. Instead of keeping the soil moist with water in the ordinary way, a dilute solution of the appropriate reagent was employed, the pots being stood in saucers to prevent loss of the reagent. Stock solutions of the substances to be used were made and stored in aspirators. Where possible the solutions were of such a strength that 10 c.c. could be added to 1 litre of water at the time of use. Water is laid on to each chamber of the glasshouse. In each a large tank is kept always full so that the water used for the plants is of an equable temperature. Water was taken from this tank for diluting the solutions. In these experiments plants were usually treated in batches of three or multiples of three. Usually 1 litre of the dilute fluid was divided roughly between three pots each time they received treatment. The solutions were made up accurately but no great care was taken to divide the litre exactly as, even if it were known precisely how much of the reagent each pot received, it would be no reliable guide to the amount the plant actually takes up. From time to time throughout treatment, and for some days after the last dose was

given, small portions of leaves were taken from the plant and their cells were examined. Usually the treatment was continued until each plant had received a litre of the dilute fluid, but this was often varied as circumstances demanded. Control plants were kept in saucers and received water on each occasion that the experimental plants were treated.

This method proved to be the neatest and simplest. It has the great disadvantage that the reagent may be precipitated by other substances in the soil and consequently will never be absorbed by the plant. The effect of every reagent was tested by this method but the following alternatives were used from time to time.

(2) *Cut shoots.*

Young axillary shoots were removed from a plant, the cut being made under water to prevent the entrance of air into the plant tissues. The cut end of the shoot was then inserted into a small conical flask containing a dilute solution of the reagent. The solutions used were 1/5 to 1/50 of the strength used in method I. Cells were examined microscopically from time to time during the experiment.

(3) *Suction through cut petiole.*

The petiole of a leaf was cut under water so as to remove the lamina. The cut end of the petiole was immediately immersed in a small tube containing a dilute solution of the reagents. The solution was drawn back through the petiole into the vascular system of the stem and thence to other tissues.

This method was not a very satisfactory one. If a relatively strong solution were used it killed the tissue of the petiole so that the cells became plugged and absorption ceased. If weaker solutions were used they became very dilute before reaching the tegumentary tissues on which most of the observations were made. The solutions used varied from 1/10 to 1/100 of the strength of those used in method I.

(4) *Injection.*

A very dilute solution was injected into the lamina of a leaf by means of a hypodermic syringe. It was found simplest to make the puncture through the lower epidermis at the juncture of two fine veins.

The solutions used were about 1/10 to 1/100 of the strength of those used in method I.

No positive results were obtained by injection into the petiole, due probably to the difficulty of suitably adjusting the strength of the solution.

*Results.**Formalin.*

Method I. 1 per cent. Toxic to all plants.

0.1 per cent. *Hyoscyamus niger*, lower leaves flagged, upper ones became crinkled. In *Hyoscyamus niger*, *Solanum nodiflorum* and *Nicotiana tabacum*, after two days the cytoplasm was found to be streaming very rapidly. This continued for some days.

0.05 per cent. *Hyoscyamus niger* leaves showed slight crinkling. Microscopic effects same as with 0.1 per cent. solution in all test plants used.

Method IV. 0.005 per cent. Cytoplasm of *Solanum nodiflorum* flowed very rapidly in cells neighbouring seat of injection.

Chloroform.

Method I. 1 per cent. *Solanum nodiflorum*, *S. lycopersicum* and *Nicotiana tabacum*. The cytoplasm was found to be streaming very rapidly. It also became very conspicuous and later whole strands seemed to become more viscous, but there was no indication of any localised coagulation (Plate XXIII, fig. 4).

2 per cent. This solution was toxic to *Solanum nodiflorum*, the lower leaves dying off rapidly. Other hosts behaved as with the weaker solution.

Ethyl alcohol.

Method I. 2 per cent. In the hairs of *Solanum nodiflorum*, *Nicotiana tabacum* and *Hyoscyamus niger*, the cytoplasm streamed with increasing rapidity, also becoming very conspicuous.

4 per cent. In the same range of plants, coagulation of the cytoplasmic strands followed accelerated streaming.

Method II. 0.5 per cent. Results as with method I.

Method IV. 0.1, 0.5 per cent. *Solanum lycopersicum*. Increased rate of streaming in neighbouring hair cells.

Urea.

Method I. 0.1 per cent. (pH=7). *Solanum nodiflorum*. Leaves assumed a very dark green colour. There was accelerated streaming of the cytoplasm which became more conspicuous. Treatment was continued in the same plants until 2 litres of solution had been added to each. No further abnormalities resulted.

Acetic acid.

Method I. 0.1 per cent. *Solanum nodiflorum*, *Nicotiana tabacum*, *Hyoscyamus niger*. After 12 hours, coagulation was observed. Plants wilted and died immediately.

0.02 per cent. Same hosts. Accelerated streaming and increased conspicuousness of cytoplasm.

Method II. 0.002 per cent. *Solanum nodiflorum*. Accelerated cytoplasmic streaming in the hair cells.

Lactic acid.

Method I. 0.1 per cent. *Solanum nodiflorum*, *S. lycopersicum*, *Hyoscyamus niger*. The cytoplasm became very conspicuous, it increased in bulk and rapidity of movement. This was especially noticeable in *H. niger*, where the movement contrasted strikingly with the usually rather sluggish streaming of the plasm in the normal plant.

0.2 per cent. Same hosts. As before, the cytoplasm increased in bulk and flowed more rapidly about the cell. Occasionally a hair cell was found in which one small portion of the cytoplasm had become slightly more conspicuous than the rest (Plate XXIV, fig. 2). As the plasm flowed about the cell, this small portion was carried along with it. No limiting membrane could be observed, but this little globule of cytoplasm retained its own identity even when buffeted by converging streams of plasm so as to become misshapen. These bodies were in appearance somewhat similar to the X-bodies of tobacco mosaic virus. They were, however, comparatively rare, and cells containing them difficult to find. When found, these bodies could usually be observed for an hour or so. Then they were suddenly lost to view, having apparently been re-absorbed into the general mass of the plasm.

In all, thirteen *Solanum nodiflorum*, three *S. lycopersicum* and two *Hyoscyamus niger* received this treatment of 1 litre of 0.2 per cent. lactic. The bodies were found in nine of the *Solanum nodiflorum* and in one tomato, but they were not found in *Hyoscyamus niger*. 0.4 per cent. solution was tried on three *Solanum nodiflorum*. No bodies could be found. It is possible that they did not form, as the cytoplasm seemed to become generally more viscous.

Method III. 0.01 per cent. *Solanum nodiflorum*. Petiole shrivelled. 0.001 per cent. *S. nodiflorum*. Accelerated streaming of the cytoplasm was noticed in some of the cells of the leaves above the one used to introduce the solution. No bodies could be found.

Method IV. 0.001 per cent. *Solanum nodiflorum*, *Nicotiana tabacum*.

In some hairs immediately surrounding the puncture, the cytoplasm seemed to have become generally coagulated. It was very conspicuous and movement was very sluggish.

Picric acid.

Method I. 0.025 per cent. *Solanum nodiflorum*, *S. lycopersicum* and *Hyoscyamus niger*. Yellow spotting of leaves.

0.0125 per cent. Same hosts. There are often a few chloroplasts in the hairs of normal plants. These seemed to increase in number. There was very little difference from usual in the appearance of the cytoplasm.

Sodium pyruvate.

Method I. 0.1, 0.2 per cent. *Solanum nodiflorum*. There was no visible effect on the cells.

Nicotine.

Method I. 0.001 per cent. *Solanum nodiflorum*, *S. lycopersicum*, *Hyoscyamus niger*. A mottle was often produced on two or three leaves of the plant. Microscopically, these leaves did not differ at all from those which retained their normal appearance. The rate of streaming of the cytoplasm was greatly increased. Minute hyaline spheres, which seemed to have a slightly different consistency from the rest of the cytoplasm, made their appearance in it and were carried rapidly about the cell.

0.005 per cent. *Solanum nodiflorum*. There appeared to be considerable coagulation in the cytoplasm.

Hyoscyamus extract.

Method I. 0.002, 0.005 per cent. *Solanum nodiflorum*, *S. lycopersicum*, *Hyoscyamus niger*. No effects were visible in the cells.

Cannabis extract.

Method I. 0.005, 0.01 per cent. *Solanum nodiflorum*. No microscopic effects were visible.

Strychnine.

Method I. 0.01, 0.05 per cent. *Solanum nodiflorum*. The usual initial stimulation of the cytoplasm preceded a slight degree of general coagulation.

Method IV. 0.005 per cent. *Solanum nodiflorum*. It was possible to induce some slight thickening of the cytoplasm in the cells of hairs near the puncture.

442 *Intracellular Inclusions in Plant Virus Diseases*

Iodine.

Method I. 0.05 per cent. Toxic.

0.01 per cent. *Solanum nodiflorum*, *S. lycopersicum*, *Hyoscyamus niger*. Apart from an initial stimulation of the cytoplasm, no cytological effects were visible.

Copper sulphate.

Method I. 0.01 per cent. (pH=5.4). *Solanum nodiflorum*, *S. lycopersicum*, *Hyoscyamus niger*. Stimulation of the cytoplasm was followed by a slight apparent increase in viscosity.

Barium chloride.

Method I. 0.1 per cent. *Solanum nodiflorum*, *S. lycopersicum*, *Nicotiana tabacum*. No effects visible microscopically.

Zinc chloride.

Method I. 0.01 per cent. *Solanum nodiflorum*. No microscopically visible effects.

Mercuric chloride.

Method I. 0.01, 0.02 per cent. *Solanum nodiflorum*, *S. lycopersicum*, *Hyoscyamus niger*. Some slight stimulation of the plasm was followed by an increase in viscosity.

Iron alum (ferrous ammonium sulphate).

Method I. 0.1 per cent. *Solanum nodiflorum*, *S. lycopersicum*, *Hyoscyamus niger*. Plants became very brittle. The streaming of the cytoplasm was increased. Treatment was continued until each plant had received 2 litres of solution. Some coagulation was then produced in the cells of the hairs.

Aluminium citrate.

Method I. 0.05 per cent. *Solanum nodiflorum*. No effect was visible in the cells of the hairs.

Lead nitrate.

Method I. 0.05, 0.1 per cent. *Solanum nodiflorum*. Early stimulation of the streaming cytoplasm was the only visible effect produced in the plant cells.

Nickel chloride.

Method I. 0.1 per cent. *Solanum nodiflorum*. The cytoplasm was stimulated to stream more rapidly. Later, some slight coagulation was brought about in the cells of the leaf hairs.

Potassium arsenate.

Method I. 0.01, 0.02 per cent. *Solanum nodiflorum*, *S. lycopersicum*, *Hyoscyamus niger*. The cytoplasm appeared to increase in bulk in all the plants. In *Solanum nodiflorum*, small particles were seen in the plasm. Later, some degree of coagulation of the cytoplasm occurred.

Ammonium molybdate.

Method I. 0.1 per cent. ($\text{pH}=4.4$). Initial observations were made on *Solanum nodiflorum*, *S. lycopersicum*, *Nicotiana tabacum* and *Hyoscyamus niger*, but later work was confined to the first-named species. This reagent produced very striking macro- and microscopic effects on all four hosts. It caused stunting of the plants and the leaves became bleached. These two symptoms varied in degree with the quantity of reagent received by the plant. One litre of 0.1 per cent. solution was sufficient to bleach completely all the leaves of a *Solanum nodiflorum* plant which was about 9 in. high at the time of treatment. $1\frac{1}{2}$ litres of this solution was completely toxic. Microscopic effects and slight macroscopic symptoms were produced by a litre of 0.025 per cent. solution. After treatment ceased the plants commenced to grow out of the effects. Yellowish green leaves were produced and sometimes mottled ones. This mottle bore very little resemblance to one due to virus infection, but it was typical of the presence of some toxic substance. Plants which had been treated never became entirely normal again. The lamina of the leaf of a normal plant is about 4 in. long, that of a treated plant seldom exceeded half this length. The whole habit of the plant can be changed by this treatment. *S. nodiflorum*, as grown in our glasshouses, produces first a single upright stem. When this is about 2 ft. high, small axillary branches grow out. Treated plants branched profusely, the secondaries being indistinguishable from the main stem. The internodes were much shorter than in the normal (Plate XXV, fig. 1). About two months after treatment, the plants bore a strong superficial resemblance to *S. nigrum*. They had the same trailing habit. The main difference lay in the form of the leaves, those of treated *S. nodiflorum* plants retained their entire margin whilst those of *S. nigrum* are coarsely toothed.

A *S. nodiflorum* plant treated in September was kept over the winter. In February, a few leaves showed a mottle resembling a virus symptom. It was thought that the plant had become accidentally infected, but it was impossible to transmit the mottle by needle inoculation. Attempts have been made to produce this mottle again. Two plants, one six and one eight months after treatment, have shown isolated mottled leaves

(Plate XXV, fig. 2). This mottle could not be transmitted by needle inoculation into *S. nodiflorum* or *Hyoscyamus niger* nor by rubbing leaves of *Nicotiana glutinosa* with juice from an affected leaf. The material obtained was insufficient to make any other transmission tests possible. It seems unlikely that the mottle was due to an accidental infection with a virus, for if a virus were present the symptoms would probably have been more general.

Ammonium molybdate treatment produced curious changes in the cells of the plants. Soon after treatment began the cytoplasm was seen to be moving very rapidly around and about the cell and it seemed to increase in bulk (cf. Plate XXIII, fig. 4). Minute particles appeared in the plasm which carried them about the cell. The particles gradually agglomerated until a single large mass of them was formed. This mass persisted in the cell as a yellowish granular body (Plate XXIV, figs. 5 and 6). These bodies were very abundant in the tissues. They were present in practically every hair cell, were abundant in the epidermis and also occurred in the palisade tissue. The plants still continued to produce them in new tissue, six months after treatment ceased.

Morphologically these bodies appeared exactly similar to those produced by aucuba mosaic and Hy. III diseases and they were formed in a precisely similar way. It will, however, be seen that they are of a different chemical nature.

It was suggested, as the salt was applied to the roots of the plant, that it might not reach the shoot and that the formation of inclusion bodies was due to some secondary effect. At this time no satisfactory microchemical tests had been devised and hence some spectrographic analyses were made by Dr S. Judd Lewis. The molybdenum content of normal leaves, of leaves from plants infected with aucuba mosaic disease and from plants treated with ammonium molybdate were analysed. Molybdenum was found to be abundant in the leaves of treated plants.

It has been generally thought that molybdenum was not usually present in plant ash (13). In view of recent work by ter Meulen (11), who has examined coal ash and also the ashes of a number of widely different plants and has invariably demonstrated the presence of molybdenum, and of the work of Roach (12), who is determining the exact significance of this element in the development of certain apple grafts, it is of interest to note that this element was also present in both normal and virus diseased plants of *Solanum nodiflorum*. Healthy leaves contained approximately 0.015 per cent. molybdenum relative to the calcium content

and the diseased leaves showed 0.011 per cent. The calcium content of the ash was not accurately determined, but it is about 10 per cent., so that the amount of molybdenum present represents a little over 0.001 per cent. of the ash.

Microchemical methods were later devised to test these inclusion bodies. They are thought to contain a certain amount of protein, as a pink coloration is produced in them by warming a strip of living tissue with Millon's reagent; with ammonia and nitric acid, they assume a deeper yellow colour. The thickness of the cell wall often prevents penetration by less strong reagents so that difficulty is experienced in obtaining satisfactory results. A method of micro-incineration is therefore being developed. The apparatus at present available is somewhat crude, an ordinary electric furnace with a refractory clay lining being used. Although there may be some cracking of the thicker cell walls, sufficiently good preparations have been obtained. Paraffin sections could not be used owing to the difficulty of finding fixatives which would neither distort unduly, nor would dissolve out, nor add, mineral substances. Frozen sections and hand sections and also strips of epidermis torn from the leaves were heated until a temperature of 550° C. was attained. This took 60 minutes. On cooling, each preparation was covered with a $2 \times \frac{7}{8}$ in. glass slip which was sealed with paraffin wax. After examination with dark ground illumination when the inclusions were seen to be of a blue colour, the sections were used for chemical tests. The wax was scraped from one end of the slip and a bubble of reagent was placed on the slide at this end. At the far end of the slip a hole was made in the wax to allow escape of air and entrance of the solution. This hole was small to prevent a sudden flooding of the slide which might displace the ash. The section was watched as the reagent passed over it. In this way the inclusion bodies were shown to contain molybdenum as they invariably gave a red coloration with a xanthate solution.

The molybdenum evidently enters into combination with some of the mineral or organic constituents of the cell, but the nature of the compound which is formed is at present unknown. The presence of phosphorus was tested for but it could not be demonstrated.

Method II. 0.001–0.01 per cent. *Solanum nodiflorum*. Bodies similar to those produced by method I were formed in the cells of the leaves in about two days.

Method III. 0.005 per cent. *Solanum nodiflorum*. Inclusion bodies were produced in a few leaves above the treated one.

Method IV. 0.01 per cent. *Solanum nodiflorum*. After repeated

attempts the formation of rather indefinite bodies was induced in cells near the region of the puncture.

The results obtained with ammonium molybdate suggested the employment of a number of other substances. It was not possible to use molybdic acid as this is almost insoluble. An account follows of experiments with two other salts of molybdic acid and also a number of compounds containing the elements of the same group as molybdenum in the periodic system.

Sodium molybdate, potassium molybdate.

Method I. 0.05 per cent. *Solanum nodiflorum*. Inclusion bodies were formed in cells just as with ammonium molybdate.

Sodium tungstate.

Method I. 0.1 per cent. (pH=6.6). *Solanum nodiflorum*. This reagent first stimulated and later caused some general coagulation of the cytoplasm.

Uranium acetate.

Method I. 0.1 per cent. *Solanum nodiflorum*. Slight stimulation was followed by general coagulation of the cell contents.

Selenious acid.

Method I. 0.1 per cent. Toxic.

0.01 per cent. *Solanum nodiflorum*. Plants became very stunted, and very small white leaves were formed as after treatment with the salts of molybdic acid. In the plant cells, slight stimulation was followed by coagulation. Numerous strands of cytoplasm passed across the vacuole of the cell.

Potassium dichromate.

Method I. 0.1 per cent. Toxic.

0.01 per cent. *Solanum nodiflorum*. Stimulation of cytoplasmic streaming was followed by an apparent increase in viscosity of the plasm.

Chromic acid.

Method I. 0.1–0.05 per cent. *Solanum nodiflorum*, *Nicotiana tabacum*, *Hyoscyamus niger*. There was a great increase in the rate of streaming of the cytoplasm. Small, spherical, hyaline bodies appeared in the cytoplasm. These minute bodies then disappeared and all the cytoplasm seemed to increase in viscosity.

Ammonia.

Method I. 0.1 per cent. *Solanum nodiflorum*. This reagent stimulated the cytoplasm to increased activity which was later followed by slight coagulation.

0.1 per cent. ammonium chloride produced similar effects.

ATTEMPTS TO INHIBIT THE DEVELOPMENT OF INCLUSION BODIES
IN VIRUS-DISEASED PLANTS.

Methods.

The method of application of the reagents was essentially that of method I (p. 437), but a much larger number of plants was involved for each experiment. For each reagent used, two series of plants were set up, one series receiving the reagent only, the second series being also inoculated with the virus. Two series of controls were also set up, one receiving water only, the second receiving water but being inoculated with the virus at the same time as the experimental plants.

The time of inoculation with the disease was varied in the different experiments. Relative times of inoculation and treatment were suggested by the periods required for microscopic symptoms of the disease and for the cytological effects of the reagents to become apparent. Therefore some inoculations were made at the time the first dose of reagent was given; others were made during the course of the treatment.

Experiments with Solanaceae.

Solanum nodiflorum and *Nicotiana tabacum* were the host plants used in these experiments. Attempts were made to inhibit the development of inclusion bodies usually produced in these hosts by aucuba mosaic disease and by tobacco mosaic disease.

The strength of the reagents to be used was determined by trial. The following were selected:

Salicylic acid. 0.02 per cent. Each plant received $\frac{1}{2}$ litre in three doses.

Potassium iodide. 0.02 per cent. Each plant received $\frac{1}{2}$ litre in three doses.

Potassium bromide. 0.05 per cent. Each plant received 1 litre in three doses.

Ammonium thiocyanate. 0.05 per cent. Each plant received $\frac{1}{2}$ -1 litre in three to six doses.

Barbitone. 0.01 per cent. Each plant received 1 litre in six doses.

The results of these experiments were disappointing. Although after treatment the cytoplasm of the uninoculated plants appeared much less

conspicuous, in all the inoculated plants inclusion bodies developed. The only noticeable effect was a retardation of symptoms in the plants treated with ammonium thiocyanate. It was thought that this ill success might be due to the acidity of the cell sap, as peptising agents often function best in alkaline media. Most plant juices lie in the acid range; this is true of all the Solanaceae used in these studies. It became necessary therefore to find a plant with an alkaline juice which would produce inclusion bodies on infection with a virus disease.

Experiments with Cucumis sativus.

B. N. Uppal, whilst working in this department, found the juice of the cucumber to be definitely alkaline. The most common virus disease of cucumber is the cucumber mosaic disease, but this produces no intracellular inclusions. It has been suggested that the development of inclusion bodies may be dependent on the pH of the plant tissue (15), and as the mosaic disease produces no inclusions in the cells of cucumber, it might be thought that their development is inhibited by the alkalinity of the sap. However, Hoggan failed to find inclusions in a number of other plants with acid juice which are susceptible to this disease (8). The writer has also failed to find inclusion bodies in *Solanum nodiflorum*, *Nicotiana tabacum*, *N. glutinosa*, *Hyoscyamus niger* and *Zinnia* infected with cucumber mosaic.

There are very few viruses to which cucumber is susceptible. Wingard (19) described a ringspot disease of tobacco as producing systemic infection in *Cucumis sativus*, and Woods (20) has demonstrated inclusion bodies in several *Nicotiana* species infected with this disease. Dr Kenneth Smith, who had obtained the virus from Dr Price, kindly supplied the author with it.

This tobacco ringspot was inoculated into *Nicotiana tabacum* var. White Burley, *Solanum nodiflorum* and *Cucumis sativus*. Inclusion bodies were produced in the cells of these plants. They were of the "amoeboid" form similar to the X-bodies produced in many hosts by tobacco mosaic virus. In White Burley tobacco, striate material was also produced, although Woods (20) did not describe this as occurring in Turkish or Havana seed-leaf varieties of tobacco. Striate material was produced also in *Solanum nodiflorum*, but it was not formed in *Cucumis sativus*. In the latter, only a very small proportion of the cells contained inclusion bodies. Plants were usually inoculated on the cotyledons when the first leaf was open. A violent chlorosis was produced on the second and third leaves. It was in the cells of these two leaves that inclusion bodies were

occasionally found. Although the plants remained stunted they did to a certain extent grow out of the effects of the virus. Later leaves showed little chlorosis and enations developed on the lower surface. No intracellular inclusion bodies were ever found in these later leaves. It will be seen that cucumber infected with tobacco ringspot did not promise to be a very favourable subject for a study of the effects of peptising agents on virus-diseased material. However, in the absence of more favourable material, several experiments were made.

The reagents used were those described above, but it was usually found necessary to reduce the dose to one-half of that given to the Solanaceous hosts. In different experiments, inoculations were made on the day treatment commenced, and on one or two days after.

The *pH* of the juice of the second and third leaves of the control plants was estimated by the quinhydrone method: it was found to be 7.6.

Again no positive results were obtained. Inclusion bodies were found in at least one inoculated plant of each series. The bodies were found very infrequently, but owing to their comparative rarity in control plants, it was not possible to determine whether any of the treatments had brought about any diminution in the number of cells so affected.

DISCUSSION.

THE DIAGNOSTIC VALUE OF INTRACELLULAR INCLUSIONS.

The possible value of the cytological characteristics of diseased tissue in the diagnosis and classification of viruses has often been discussed. It is obviously not possible to identify a particular virus disease by these features alone, although each virus seems to be consistent in the type of inclusion it produces in a range of host plants. Both Hy. III and aucuba mosaic disease invariably cause the appearance in the cytoplasm of small particles which aggregate and fuse, the mature body, however, being slightly modified in form by different hosts. The bodies produced by these two diseases are indistinguishable morphologically, but they differ greatly from the amoeboid X-bodies of tobacco mosaic. The latter also appear always to be accompanied by striate material. Ringspot disease seems invariably to produce similar amoeboid bodies, whilst cucumber mosaic apparently causes no cytological irregularities. It would thus appear that the presence or absence of intracellular inclusions and their morphology when present should be of very considerable value in supplementing other diagnostic features.

THE NATURE OF INTRACELLULAR INCLUSIONS.

In a series of experiments such as those described in this paper, the possible variations in detailed conditions are almost infinite, but in each experiment the correct combination of conditions must be arrived at before the desired results can accrue. Available data on which to base experimental methods is particularly scanty, and consequently in this rather cursory study favourable results had of necessity to depend largely on the chance selection of the proper procedure. The plants responded to a certain extent to most of the coagulating agents. Almost without exception, these reagents induced an increased activity of the cell contents which flowed more freely whilst becoming more conspicuous. This effect is probably similar to that of drugs, which in small doses will stimulate, but it is also analogous to the action of certain viruses which, prior to the formation of inclusion bodies, stimulate the cytoplasm to increased activity. It is probable that more detailed investigations of many of these reagents would result in the production of further abnormalities. Throughout the experiments very little regard was had to either the *pH* or to the osmotic pressure of the solutions used. Although possibly of paramount importance, by method I these factors could not in any way be controlled, as the result of contact with the soil could not be estimated. Thus the negative results recorded here can be regarded as in no way conclusive.

With regard to the work on coagulants, the main object has been attained. It has been found possible, in the absence of the etiological agent of the disease, to parallel all the cytological phenomena induced by a virus. The salts of molybdic acid induced changes in the cell contents similar to those produced by aucuba mosaic and by Hy. III diseases. Soon after treatment the cytoplasm flowed more rapidly about the cell, then minute particles became evident and were carried passively about the cell by the cytoplasmic stream. These particles fused together until all were agglomerated into a single large body. The mode of formation of inclusion bodies in aucuba mosaic and in Hy. III diseases suggests them to be coagulation products of the cytoplasm, a view which is supported by the fact that similar changes can be wrought in the cytoplasm by physico-chemical experimentation.

Treatment with lactic acid occasionally causes a minute portion of the cytoplasm of a cell to become slightly differentiated from the rest in such a way that it could remain intact for some hours as it was carried about the cell and buffeted by the streaming plasma. These bodies are

in appearance very like the X-bodies of tobacco mosaic disease, suggesting that the latter, although differing in appearance from the inclusions of aucuba mosaic disease, are of essentially similar origin, for both types of body can be artificially induced in healthy cells by similar treatments.

The substance of the inclusion bodies appears to consist of material precipitated or coagulated from the cell sap or cytoplasm due either to the action of the virus itself or to some substance or a change in physical conditions produced by unbalanced metabolism resultant on virus infection. Distinction has been drawn between the rounded inclusion bodies and the more crystalline structures such as the striate material of tobacco mosaic disease. These two types of inclusion may differ in chemical nature as do probably inclusions produced by different viruses or possibly those produced by the same virus in different hosts. But their origin would seem to be essentially similar. The fact that the striate material does in some cases fuse into a single mass lends further support to this view.

Of the results of the experiments designed to inhibit the development of inclusions little can be said, except to emphasise the difficulty of adequately controlling conditions within the cell. It is hoped that new methods of approach may bring more conclusive results.

SUMMARY.

The intracellular changes resultant on infection with aucuba mosaic and Hy. III diseases are described and are compared with the cytological effects of tobacco mosaic virus. With the two former viruses, inclusion bodies are formed by the aggregation and fusion of minute particles which appear in the cytoplasmic stream. With tobacco mosaic disease an amoeba-like body is produced and this persists for some weeks before suddenly disappearing again. It is accompanied by striate material all of which ultimately fuses into one large body.

Attempts have been made to parallel these conditions in healthy cells of Solanaceous plants by treatment with substances known to coagulate protoplasm. Almost all the reagents used induced stimulation of the cytoplasmic stream similar to the initial sign of virus infection. With salts of molybdic acid, all the cytological abnormalities due to aucuba mosaic or Hy. III disease have been imitated. Treatment with lactic acid induces the formation of amoeboid bodies like the X-bodies of tobacco mosaic, but these bodies persist for only a few hours.

Attempts have also been made to inhibit the formation of inclusion bodies induced by several different diseases in a number of hosts but no success was obtained.

The experiments support the view that the intracellular inclusions of plant virus diseases are essentially products of the host cell.

REFERENCES.

- (1) BANCROFT, WILDER D. and RICHTER, G. H. (1931). Colloid chemistry of insanity. *Journ. Physic. Chem.* xxxv, 1606-23.
- (2) BANCROFT, WILDER D. and RUTZLER, J. E. (1931). Colloid chemistry of the nervous system. Sodium thiocyanate therapy. I. *Ibid.* xxxv, 1185-1211.
- (3) ——— (1932). Irritability and anaesthesia in plants. *Ibid.* xxxvi, 273-85.
- (4) BANCROFT, WILDER D. and co-workers (1931-2). Series of papers on "Reversible coagulation in living tissue." *Proc. Nat. Acad. Sci.* xvii-xviii.
- (5) GOLDSTEIN, BESSIE (1926). A cytological study of the leaves and growing points of healthy and diseased tobacco plants. *Bull. Torr. Bot. Club*, liii, 499-599.
- (6) ——— (1927). The X-bodies in the cells of dahlia plants affected with mosaic disease and dwarf. *Ibid.* liv, 285-93.
- (7) HAMILTON, MARION A. (1932). On three new virus diseases of *Hyoscyamus niger*. *Ann. App. Biol.* xix, 550-67.
- (8) HOGGAN, ISMÉ A. (1927). Cytological studies on virus diseases of Solanaceous plants. *Journ. Agric. Res.* xxxv, 651-71.
- (9) IWANOWSKI, D. (1903). Ueber die mosaikkrankheit der Tabakspflanzen. *Zeitsch. Pflanzenkr.* xiii, 1-41.
- (10) KUNKEL, L. O. (1924). Histological and cytological studies on the Fiji diseases of sugar cane. *Hawaiian Sugar Planters' Assoc. Bull. Exp. Sta. Bot. Ser.* iii, 99-107.
- (11) TER MEULEN, H. (1932). Distribution of molybdenum. *Nature*, cxxx, 966.
- (12) ROACH, W. A. (1933). Distribution of molybdenum. *Ibid.* cxxxi, 202.
- (13) ROBINSON, W. O., STEINKOENIG, L. A. and MILLER, C. F. (1917). The relation of some of the rarer elements in soils and plants. *U.S. Dept. Agric. Bull.* No. 600.
- (14) SHEFFIELD, F. M. L. (1931). The formation of intracellular inclusions in Solanaceous hosts infected with aucuba mosaic of tomato. *Ann. App. Biol.* xviii, 471-93.
- (15) ——— (1933). The development of assimilatory tissue in Solanaceous hosts infected with aucuba mosaic of tomato. *Ibid.* xx, 57-69.
- (16) ——— (1933). Virus diseases and intracellular inclusions in plants. *Nature*, cxxxi, 325-6.
- (17) SMITH, J. HENDERSON (1930). Virus diseases in plants. II. The amoeboid intracellular inclusions. *Biol. Rev.* v, 164-70.
- (18) SMITH, KENNETH M. (1933). *Recent Advances in the Study of Plant Viruses*. London: J. and A. Churchill.
- (19) WINGARD, S. A. (1928). Hosts and symptoms of ringspot, a virus disease of plants. *Journ. Agric. Res.* xxxvii, 127-53.
- (20) WOODS, MARK W. (1933). Intracellular bodies associated with ringspot. *Contrib. Boyce Thompson Inst.* v, 419-34.

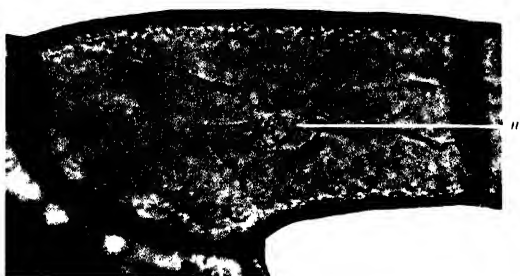


Fig. 1.

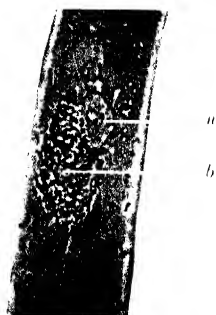


Fig. 3.

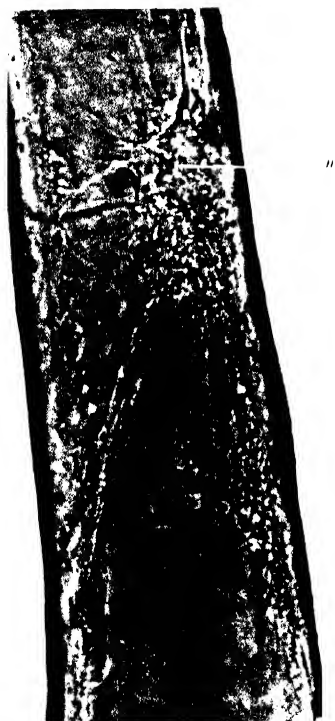


Fig. 2.

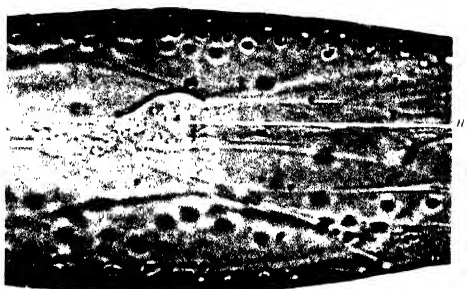


Fig. 4.

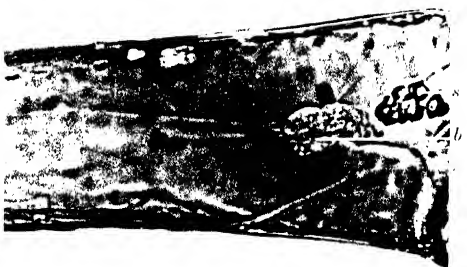


Fig. 5.



Fig. 1.

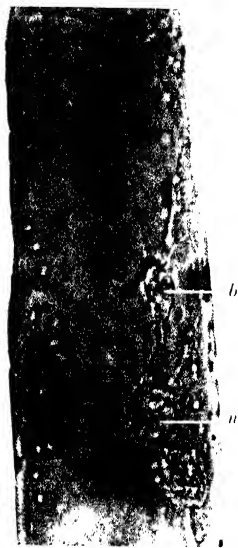


Fig. 2.



Fig. 3.



Fig. 4.



Fig. 5.



Fig. 6.



Fig. 1.



SHEFFIELD.—EXPERIMENTS BEARING ON THE NATURE OF INTRACELLULAR INCLUSIONS
IN PLANT VIRUS DISEASES (pp. 430-453).

EXPLANATION OF PLATES XXIII—XXV.

Some photomicrographs were taken with a Leitz Micca, others with a Leitz Makam camera. A Leitz 6L objective was used in combination with a Leitz periplanat eyepiece either $\times 8$, $\times 10$, or $\times 12$. Photographs taken with the Micca camera were enlarged three times for reproduction; those taken with the Makam have been reproduced unaltered.

All photomicrographs were taken from unstained living cells.

The following symbols are used to label the figures: *b* = intracellular inclusion body; *n* = cell nucleus; *s* = striate material.

PLATE XXIII.

- Fig. 1. *Solanum nodiflorum*. Basal cell from hair of normal healthy plant. The nucleus is suspended in the centre of the cell by a few delicate cytoplasmic strands. $\times 450$.
 Fig. 2. *Nicotiana tabacum*. Hair cell a few days after infection with tobacco mosaic disease. The cytoplasm is much more conspicuous than in the normal cell and appears to have increased in bulk. $\times 525$.
 Fig. 3. *Nicotiana tabacum*. Inclusion body produced in cell of trichome as a result of infection with Hy. III disease. $\times 450$.
 Fig. 4. *Solanum nodiflorum*. Cell from hair soon after treatment with chloroform. The cytoplasm has become more conspicuous and has increased in bulk. $\times 450$.
 Fig. 5. *Nicotiana tabacum* with ring spot disease. An amoeboid inclusion body and striate material have formed in the hair cell. $\times 450$.

PLATE XXIV.

- Fig. 1. *Nicotiana tabacum* with tobacco mosaic disease. An amoeboid X-body and striate material are present in the hair cell. $\times 500$.
 Fig. 2. *Solanum nodiflorum* after treatment with lactic acid. An inclusion, morphologically similar to the X-bodies of Plate XXIII, fig. 5 and Plate XXIV, fig. 1, is present in this hair cell. $\times 450$.
 Fig. 3. *Solanum nodiflorum*. Six weeks after infection with tobacco mosaic disease, the amoeboid X-body has disappeared and the striate material has formed a single large mass. $\times 400$.
 Fig. 4. *Solanum nodiflorum*. Inclusion body produced in cell of trichome on infection with aucuba mosaic disease. $\times 450$.
 Figs. 5 and 6. *Solanum nodiflorum*. Inclusion bodies morphologically similar to those of Plate XXIII, fig. 3, and Plate XXIV, fig. 4, are produced in cells as a result of treatment with ammonium molybdate. $\times 400$.

PLATE XXV.

- Fig. 1. *Solanum nodiflorum*. On the right, a healthy plant two months old showing the normal erect habit. On the left, a plant five months after treatment with ammonium molybdate. This plant is much branched, it has a trailing habit and small leaves. A few of the older leaves show the initial typical poison mottle.
 Fig. 2. *Solanum nodiflorum*. Branch of plant six months after treatment with ammonium molybdate. One leaf bears a mottle similar to that produced by a virus. All other leaves of the branch are a uniform yellowish green colour. $\times 1\frac{1}{2}$.

(Received April 2nd, 1934.)

The Constancy of the Viscosity of Strong Lithium Chloride Solutions at low Velocity Gradients. By G. W. SCOTT BLAIR, M.A., and R. K. SCHOFIELD, M.A., Ph.D. (Physics Department, Rothamsted Experimental Station, Harpenden, Herts, England).

DATA were presented in an earlier paper[‡] which seemed to show that a strong solution of lithium chloride, when forced through a narrow tube, does not flow in exact conformity with Poiseuille's law. Parallel data for a glycerine-water mixture, having a similar viscosity,

[‡] G. W. Scott Blair and R. K. Schofield, *Phil. Mag.* xi. p. 890 (1931).

revealed no anomaly, and therefore appeared to rule out the possibility that the discrepancies observed when using the lithium chloride solution were due to imperfections in the apparatus. The subsequent publication by Ostwald and Malss* of data for a lithium chloride solution in which no anomaly is apparent led us to repeat and extend our earlier measurements. Using the same apparatus as before, anomalous flow was confirmed with several solutions of lithium chloride, and was also observed for strong solutions of potassium carbonate and magnesium chloride. Regular behaviour was again found when using glycerine-water mixtures, and, as a further check on the apparatus, mixtures of medicinal paraffin (kerosene) and benzene were used, and no anomaly was found.

An attempt was next made to demonstrate the inconstancy of the viscosity of strong salt solutions by an entirely different method. Two brass cylinders were drilled axially, and threaded on to a thin vertical rod attached by its upper end to a steel torsion wire, the system having a period of 28 sec. The lower cylinder (1.1 cm. radius and 7.5 cm. long) was surrounded by the solution contained in a wide boiling tube (3 cm. diameter), while the upper one (3.7 cm. radius and 7.7 cm. long) served to increase the inertia of the system. A scale of degrees was mounted on the large cylinder and observed through a low-power microscope with an eye-piece cross-wire. Rotational oscillations were started, and the logarithmic decrement of the amplitude determined. Even though the experiments were continued until the amplitude had fallen to half a degree (maximum velocity gradient 0.003 sec.^{-1}), no variation in the logarithmic decrement was found exceeding what would be caused by an error of 0.1° in any single reading (*cf.* Table I.). This result, therefore, confirms and extends those of Ostwald and Malss in which the velocity gradient at the wall for the lowest stress used was 0.6 sec.^{-1} .

This being the case, the discrepancies observed with our capillary tube apparatus must be due to some imperfection in the instrument, though it is evident that the defect is a subtle one, seeing that it is selective—always appearing with strong salt solutions, and never with glycerine-water or paraffin-benzene mixtures.

* Wo. Ostwald and H. Malss, *Koll. Zeits.* lxiii. p. 61 (1933).

TABLE I.

1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11
Swing no.	Double amplitude (degrees).	Swing no.	Double amplitude (degrees).	Column 2 $\times 0.3253$.	Swing no.	Double amplitude (degrees).	Column 2 $\times (0.3253)^2$.	Swing no.	Double amplitude (degrees).	Column 2 $\times (0.3253)^3$.
1	71.7	41	23.3	23.3	81	7.6	7.6	121	2.4	2.5
6	62.2	46	20.2	20.2	86	6.8	6.6	126	2.1	2.1
11	53.8	51	17.4	17.5	91	5.8	5.7	131	1.8	1.9
16	46.6	56	15.2	15.2	96	5.0	4.9	136	1.6	1.6
21	40.7	61	13.2	13.2	101	4.4	4.3	141	1.4	1.4
26	35.5	66	11.5	11.5	106	3.8	3.8	146	1.2	1.2
31	30.8	71	10.1	10.0	111	3.2	3.3	151	1.1	1.1
36	26.8	76	8.6	8.7	116	2.8	2.8	156	1.0	0.9
41	23.3	81	7.6	7.6	121	2.4	2.5	161	0.8	0.8

A clue was obtained through our carrying out an experiment in which a burette was attached to one end of the capillary tube, the other end as usual being joined to a wide bulb. It was noticed that with the lithium chloride solution the zero showed a progressive fall, indicating an escape of some three cubic centimetres of fluid during a morning's experimentation. This experiment was repeated a number of times, a fall in zero *always occurring with the lithium chloride solution and never with the glycerine-water mixture*. The junctions between the capillary tube and the burette and bulb were made by grinding the ends flat and holding them together by means of sleeves of rubber tubing. The escape could only have occurred through a creep of the salt solution between the glass and the rubber. Further consideration shows that such an escape is more likely in the case of the salt solution, since, owing to its high density, it exerts a greater hydrostatic pressure although this is always below that of the thermostat water surrounding the junction. Quite apart from this, however, a chemical test showed, under equally favourable pressure conditions, a small but definite leakage in the case of lithium chloride and potassium carbonate, but none in the case of glycerine.

That the rubber junctions were responsible for the discrepancies was rendered even more probable by the complete disappearance of the anomalies when the capillary was fused on to the bulbs of the original apparatus. Data obtained for the lithium chloride solution in this way were in complete accord with Poiseuille's law. The dimensions of a capillary with ground ends can be determined more precisely than one which is fused on to a wide tube, and it was for this reason that rubber sleeve joints were originally used. The effective dimensions of a fused-in capillary can, however, be obtained by calibration with a true fluid of known viscosity. Using capillaries calibrated in this way, the deviations from the R^4 law were found as usual with a clay suspension, so that the conclusions which the authors* have drawn regarding such systems are not affected by the results here recorded.

* R. K. Schofield and G. W. Scott Blair, Journ. Phys. Chem. xxxiv. p. 248 (1930); G. W. Scott Blair, Journ. Phys. Chem. xxxiv. p. 1505 (1930); R. K. Schofield and G. W. Scott Blair, Journ. Phys. Chem. xxxv. p. 1212 (1931).

Our natural regret that we should have been led to draw unwarranted conclusions about the viscous behaviour of a strong salt solution is tempered by satisfaction in that the flow-meter*, which is the distinctive feature of our apparatus, was not to blame. In this device the pressure difference developed by allowing the air displaced by the flowing liquid to escape through a capillary tube is measured on an alcohol manometer set at one in ten. The range of the flow-meter can be altered by changing the air capillary. The flow-meter has been subjected to constant test and has proved itself thoroughly reliable. The reading of the flow-meter usually becomes steady after applying the pressure for a few seconds, so that it is very rapid in operation.

We are not aware that the peculiar ability of very strong salt solutions to "creep" between rubber and glass has been observed before, and we hope that by putting on record our own experience we may prevent others from being similarly misled.

Summary.

Measurements of the logarithmic decrement of a cylinder executing rotational oscillation while immersed in a strong lithium chloride solution revealed no inconsistency in the viscosity, even though the final amplitude was so small that the maximum velocity gradient was only 0.003 sec.^{-1} .

The data confirm and extend that obtained by Ostwald and Malss using a capillary viscometer in which the velocity gradient at the wall for the lowest stress was 0.6 sec.^{-1} .

The anomalies reported earlier by the authors appear to have been due to the ability of the strong salt solutions used to "creep" under the rubber sleeves which held the capillaries in place, a property not shared by the glycerine-water mixture used to check the standardization of the apparatus. On sealing the joints, the anomalies in the case of the lithium chloride solution disappear, but the clay pastes still show a characteristic behaviour including departure from the R^4 law.

The flow-meter used in this work has proved itself both trustworthy and convenient in operation.

* G. W. Scott Blair and E. M. Crowther, *Journ. Phys. Chem.* xxxiii, p. 321 (1929).

A PHOTOGRAPHIC MOONLIGHT RECORDER. By C. B. WILLIAMS, Sc.D. AND G. A. EMERY, B.A., Department of Entomology, Rothamsted Experimental Station

[MS. received 10th December, 1934]

ABSTRACT. The paper describes a photographic recorder for moonlight. It consists of a cylindrical lens mounted on a light-tight drum which rotates at a speed of one revolution in 24 hours and 50 min., which is the average time of the moon's apparent rotation round the earth. The axis of the drum is set pointing to the pole star and by means of a timing disk the drum is set each afternoon so that the lens follows the position of the moon. Inside the rotating drum is a fixed drum on the outer surface of which is a strip of photographic bromide paper. On this the line image of the moon, produced by the cylindrical lens, is focused. The darkening of the bromide paper gives an indication both of the duration and of the intensity of the moonlight.

IN the course of some work on the influence of climatic conditions on insect activity it became necessary to have a record of the duration of moonlight. No instrument appeared to be available to give this information, so that one had to be designed. At first photoelectric methods were considered, but the cost was found to be too high, so that a photographic instrument was finally used.

The difficulties encountered were chiefly due to the rapid changes in position of the moon, which "revolves round the sky" in an average period of 24 hours and 50 min., but may be about 20 min. shorter or longer than this average. It also changes in position from its highest angle at southing to its lowest angle every half lunar month, and is highest in the sky at full moon and lowest at new moon in December and the reverse in June.

Difficulty was originally anticipated in obtaining a photographic paper sensitive to the feeble light of the moon. This, however, was found to be quite a simple matter with the concentration of the light by means of a cylindrical lens.

PRINCIPLES OF THE INSTRUMENT

The instrument finally constructed (Fig. 1) consists in general of a plano-convex cylindrical lens of about 1 in. focal length which is rotated slowly round a fixed drum about 3 in. in diameter so that it produces a line image of the moon on a strip of photographic paper fixed to the outer rim of the drum. The axis of the instrument is directed towards the pole star, and the speed of rotation of the lens is one revolution in 24 hours and 50 min. The position of the lens is arranged each day so as to be exactly directed towards the moon when this is at south. As the total timing error in one rotation is at a maximum 20 min., and as the instrument is seldom used more than 6 hours on either side of southing, that means that the timing error of the lens to the direction of the moon is never more than 5 min., which can be neglected.

A mask is arranged close to the photographic slip so as to allow light only through the centre portion (about $\frac{1}{3}$ in.) of the line image when the moon is on a plane at right angles to the polar axis, and the light rays therefore at right angles to the straight front surface of the lens. When the rays become more oblique the line image shifts slightly up or down, but the mask still allows a portion of it to reach the same part of the photographic strip, so that the record does not shift as the moon changes its position relative to the axis of the instrument.

CONSTRUCTION

The apparatus is shown complete (except for a glass cylindrical cover to protect from rain) in Fig. 1 and in diagrammatic cross-section plan and elevation in Figs. 2 and 3. It was made from a standard meteorological one-day clock drum *a* of just over $3\frac{1}{2}$ in. in diameter, the clock-work of which was regulated to rotate at the required speed. The axis *d* was firmly fixed at the bottom to a square stout brass base *e* which can be slipped into a slot in the stand (Fig. 1).

At one side of the base is a projecting pointer *f* indicating exact south, which is of course in the middle of the uppermost edge when the instrument is in its inclined position.

On the axis just above the base is a disk free to rotate with moderate ease, on which is marked the time scale of rotation *g*. For that portion of the time scale which deals with the middle of the day it is necessary to have two scales, one for the day previous to use, and one for the day following, unless the instrument is always removed before noon and reset after noon. Above this is fixed to the axis the cog wheel *q* round which the drum rotates, and above this the drum itself, free to rotate

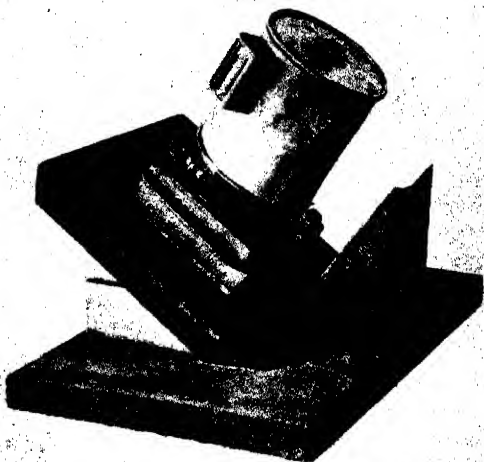


Fig. 1. Photographic moonlight recorder

by its own mechanism. To the upper portion of the drum on one side is attached an oblong projection *i* holding the lens *j* and on the opposite side a counterpoise *l* of equal weight. On the bottom rim of the drum immediately beneath the centre of the lens is a pointer *h* which comes into close proximity to the time scale. When the pointer on the drum is opposite the south indicator on the base, the lens should be facing due south and the image on the photographic strip should be exactly on the south marks on the strip (see below).

Inside the main drum, in the upper portion of it which is free from mechanism, there is fixed to the axis a three-pointed support *m* on which rests a wooden disk or inner drum *o* about $\frac{3}{4}$ in. thick and about $2\frac{3}{8}$ in. in diameter, on the outside of which the strip of photographic paper *p* is fastened. This paper should of course be at the focus of the lens. Between the lens and the inner drum and as near to the latter as possible is the mask *k* to cut out extraneous light and to reduce the length of the image to about one-third of an inch. The side of the inner drum away from the south is cut away so as to allow the ends of the paper strip to be fastened down without anything projecting beyond the outer edge of the drum.

The drum must always be replaced in exactly the same position relative to south and this is arranged by a small projecting peg *n* on one of the arms of the support fitting into a slot

on the underside of the inner drum. In addition there are on the edge of the inner drum two very small projecting pin points on the exact south position and one other a little to one side. These pierce the paper strip when it is pressed on to the drum and leave on it a permanent record of the south position of the image and the early and later sections of the record, thereby preventing it from becoming reversed.

Some difficulty was at first experienced in getting the normal push-in joint of the lid of the drum light-tight, but this was finally achieved by lining the inside of the drum and lid with black velvet. All portions of the interior not covered with velvet, including the inner drum itself and the lens holder, are painted dead black.

The instrument when in use is placed on a base of the necessary inclination (equal to the latitude of the locality where used) which is permanently fixed in a position where there will

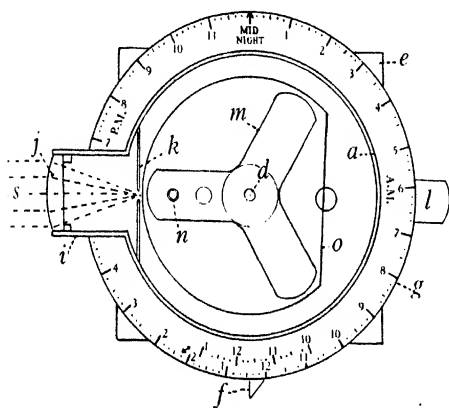


Fig. 2. Plan of moonlight recorder (half size)

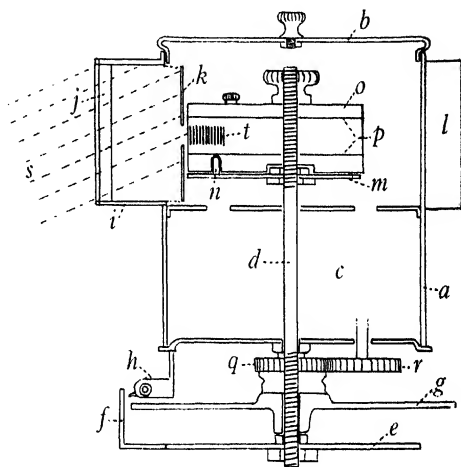


Fig. 3. Cross-sectional elevation of moonlight recorder (half size)

be no obstruction to the free view of the moon from rising to setting. It is further covered with a glass cylinder of about 7 in. diameter with a metal top, to protect it from rain and snow. There is also a small cap to cover the lens which can be put on when moving the instrument.

The photographic paper used at present is Messrs Ilford Ltd.'s normal glossy bromide. It is obtained in sheets $8\frac{1}{2} \times 6\frac{1}{2}$ in. (whole plate), and each sheet is cut longitudinally into about ten strips.

METHOD OF USE

The instrument is removed from the fixed stand and taken into a dark room. The inner drum is removed, a strip of photographic paper is fastened round its edge and it is then replaced in its correct position on the support. The lid is replaced on the instrument and the cap put on the lens. The instrument is then replaced on the stand and the timing disk rotated until the time at which the moon will south during the following night is exactly opposite the southing indicator on the base. This "southing time" can be most conveniently obtained from *Whitaker's Almanack*. The drum is next rotated till the pointer beneath the lens points to the actual time at the moment of setting on the time disk. The cap is then removed from the lens and the cover placed over the whole instrument.

On the following day, the pointer should first be inspected to see that it points to the correct time, the cap is then placed on the lens, the instrument slipped from the stand and taken again to the dark room where the photographic strip is developed and dated. A standard strength and time of development should be used so that the darkness of the image may give some indication of the intensity of the moonlight.

After the strip has been fixed, washed and dried it is laid on a time scale with the southing mark (the punctures of the two pins) opposite the correct time, and then the true hours of the night and time of moon rise or moon set, sun rise and sun set can be marked on and all the unnecessary portions trimmed off.

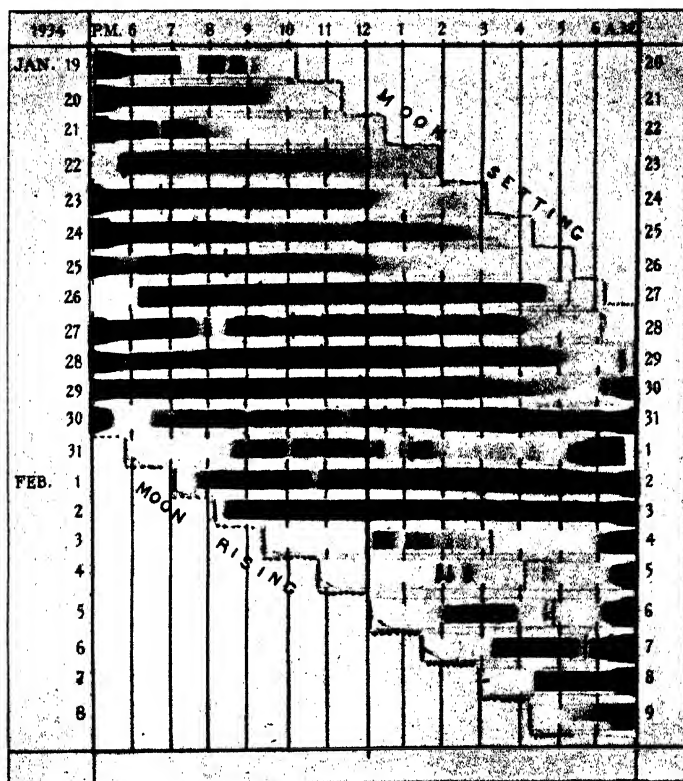


Fig. 4. Specimen records obtained ($\frac{1}{8}$ full size)

As the lens is open during day and night, there is fogging at dusk and dawn which destroys the record for about $1\frac{1}{2}$ hours after sun set and before sun rise, but of this only about $\frac{1}{2}$ hour at each period is really dark enough to be significant. The extent of the fogging is governed by the closeness of the fit of the mask against the paper.

The interpretation of the results largely depends on what use is to be made of them. By a comparison of the charts obtained from this instrument with those from a night sky recorder using the image of the pole star* it is found, as would be expected, that considerable light comes from the moon through thin clouds that obscure the pole star.

* See Introduction to *Greenwich Meteorological Observations*, 1931, pp. E. 8 and 9.

Fig. 4 shows the records obtained during a lunar month in January to February 1934. Within a few days of no moon the records are so faint, and the moon is only above the horizon so near to daylight, that the records are of little value, and the instrument is not now put out during the week of no moon.

From the figure it will be seen that to a certain extent both qualitative and quantitative information can be obtained. Thus on the night of January 19th-20th (first strip, Fig. 4) the sky was clear from 5.30 to 7.15 p.m., heavily clouded from 7.15 to 7.45, and then from 7.45 to 9.15 there were sharp-edged clouds passing across a clear sky giving a series of sharp black line images; the moon set about 10 p.m. On the 20th the sky was clear till 9 p.m. then gradually clouded over and was continuously cloudy till the moon set at 11.20. On the 21st the sky was clear with a few clouds till between 7 and 8 p.m. and then became cloudy till midnight. Other examples show that the sky was quite clear on January 26th and February 2nd; almost clear on January 28th and February 1st; there were thin clouds reducing the intensity and definition of the image on January 25th and 27th; while on February 3rd, 4th and 5th there was heavy cloud except for a few short intervals.

The instrument as described has been in use for just over one year and is sufficiently sensitive to record the light of a lamp of about 300 candle-power at a distance of over 300 yards. It has worked well enough to supply all the information that we desired, but it is possible that a larger drum might be more useful for more accurate work.

The instrument cost £2. 5s. 0d. for the clockwork drum and about 10s. for the lens; the rest of the work was done in our own workshops. The photographic paper is 2s. 7d. per dozen sheets, so that the cost is below ¼d. per night.

USE AS SUNLIGHT RECORDER

It has been found by small experiments that the instrument will work equally well as a sunlight recorder, although no continuous use has been made of it in this way. For this purpose the fast bromide paper is replaced by a slow daylight printing paper; the clock adjusted so as to make one revolution in 24 hours, and the time disk must be similarly graduated and permanently fixed with the 12 noon towards the south. There is then no need for the south indicator on the base.

Indian Agricultural Research Institute (Pusa)

LIBRARY, NEW DELHI-110012

This book can be issued on or before.....

Return Date	Return Date